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Review Article

# Immunoexcitotoxicity as the central mechanism of etiopathology and treatment of autism spectrum disorders: A possible role of fluoride and aluminum

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## Abstract

Our review suggests that most autism spectrum disorder (ASD) risk factors are connected, either directly or indirectly, to immunoexcitotoxicity. Chronic brain inflammation is known to enhance the sensitivity of glutamate receptors and interfere with glutamate removal from the extraneuronal space, where it can trigger excitotoxicity over a prolonged period. Neuroscience studies have clearly shown that sequential systemic immune stimulation can activate the brain's immune system, microglia, and astrocytes, and that with initial immune stimulation, there occurs CNS microglial priming. Children are exposed to such sequential immune stimulation via a growing number of environmental excitotoxins, vaccines, and persistent viral infections. We demonstrate that fluoride and aluminum ($Al^{3+}$) can exacerbate the pathological problems by worsening excitotoxicity and inflammation. While $Al^{3+}$ appears among the key suspicious factors of ASD, fluoride is rarely recognized as a causative culprit. A long-term burden of these ubiquitous toxins has several health effects with a striking resemblance to the symptoms of ASD. In addition, their synergistic action in molecules of aluminofluoride complexes can affect cell signaling, neurodevelopment, and CNS functions at several times lower concentrations than either $Al^{3+}$ or fluoride acting alone. Our review opens the door to a number of new treatment modes that naturally reduce excitotoxicity and microglial priming.

**Key Words:** Aluminofluoride complexes, aluminum, autism spectrum disorders, cytokines, fluoride, glutamatergic neurotransmission, immunoexcitotoxicity, microglial activation, neurodevelopment

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## INTRODUCTION

The mechanisms involved in autism spectrum disorder (ASD) etiopathology are many, but central to these disorders appears to be prolonged and repeated systemic immune activation and excitotoxicity. It has been generally accepted that chronic inflammation is the hallmark of many neurological and neurodegenerative diseases.[10,29,31,68] Studies of autistic patients have confirmed the presence of a number of immune dysfunctions.[164] Inflammation in the brain has been reported, by several groups, in postmortem brain specimens of both young and old individuals with ASD.[24,88,187,264]

The initial reaction to systemic inflammation may be microglial priming or full neurotoxic activation, depending on the intensity of the activating stimulus. It is known that a great number of conditions can lead to the priming/activation of microglia, such as stress, obesity, trauma, infections, sequential vaccination, hypoxia-ischemia, and exposure to certain neurotoxic metals, such as mercury, lead, and aluminum.

Microglia are involved in every aspect of neurodevelopment.[210,258] Full activation of primed microglia leads to a release of high levels of chemokines, cytokines, interferons, and inflammatory prostaglandins, as well as an assortment of excitotoxic amino acids, such as glutamate, aspartate, and quinolinic acid. It becomes evident that numerous cytokines and chemokines play essential roles in neurodevelopment. Significant alterations of these immune mediators during critical periods of brain development can also cause varying degrees of functional brain disruptions, resulting in abnormal connectivity and disrupted cytoarchitecture. Studies of autistic patients report elevations of particular cytokines and the presence of antibrain antibodies.[73,133,259]

There is growing evidence that glutamate and glutamate receptors (GluRs) are involved in neurodevelopment.[55,77,191] Glutamate neurotransmission far surpasses the presence of other neurotransmitters in the brain, accounting for 90% of neurotransmission in the cortex and 50% of the entire brain being glutamatergic.[286]

In 1969, Olney demonstrated that exposure of specific types of neurons to high levels of glutamate caused a delayed death process that involved intense excitation of neuronal activity (excitotoxicity).[183] Excitotoxicity is caused by excess levels of extraneuronal glutamate and overactivation of ionotropic GluRs on neuronal membranes, leading to ionic influx, disruption of energy metabolism, and potential neuronal death. In 2008, Blaylock coined the term immunoexcitotoxicity to describe the interaction of inflammatory mechanisms and excitotoxicity.[25]

In a previous article, we presented evidence that most heterogeneous symptoms of ASD have a common set of events closely connected with dysregulation of glutamatergic neurotransmission in the brain.[33] It has become accepted that the causation of ASDs represents an assortment of genetic predispositions interacting with a number of enviromental factors. Two such factors include aluminum and fluoride. Aluminum's free metal cation ($Al^{3+}$) is an experimentally demonstrated neurotoxin whose ability to impact the human nervous system has been known for decades.[23,193,257,270] While $Al^{3+}$ appears among the key suspicious factors contribution to ASD, fluoride is rarely recognized as a causative culprit of increasing prevalence of ASD.[171,239,256] Fluoride and $Al^{3+}$ are two ubiquitous contaminants of the environment, including water, nutritional substances, cosmetics, and pharmaceutical products.

In areas with fluoridated drinking water, we observe some symptoms of ASD, such as IQ deficits, sleep-pattern disturbance, inflammation, impaired ability of cognition, and learning and behavioral problems in some individuals. Prolonged exposure to fluoride in the prenatal and postnatal stages of development might have toxic effects on the development and metabolism of brain. Moreover, both fluoride and $Al^{3+}$ interfere with a number of enzymes, resulting in a significant suppression of cellular energy production and oxidative stress.[237] As pointed out in a previous papers, one must also consider interactions between these two reactive elements within biological systems.[238,240,242] Such interactions occur rapidly, forming aluminofluoride complexes ($AlF_x$), which allow them to activate hundreds of G protein-coupled receptors (GPCR) at several times lower concentrations than either $Al^{3+}$ or fluoride acting alone. Signaling disorders represent an important disturbance in ASD pathology. Recent studies have also shown that both fluoride and $Al^{3+}$ individually, as well as when combined, can activate microglia with release of proinflammatory cytokines. A careful review of known environmental and pathological links to ASD indicates that most of their effects are connected to immunoexcitotoxic processes.[23,239,240] Despite intensive research, an effective therapy of ASD symptoms supported by evidence-based medicine has not been found.[154] Our hypothesis opens the door to a number of new treatment modes, including the nutritional factors that naturally reduce excitotoxicity and microglial priming.

## MICROGLIAL ACTIVATION IN BRAIN DEVELOPMENT AND AUTISM SPECTRUM DISORDER

Microglia and astrocytes are involved in every aspect of brain development, including synaptogenesis and refinement, synaptic pruning, neuron elimination, angiogenesis, as

well as for maintenance, proliferation, differentiation, and migration of progenitor cells.[98,166,190,210,212] Microglia regulate phagocytosis of apoptotic cells, and a slow-down in proliferation and programmed cell death. Cunningham *et al*. noted that in the primate phagocytosis occurred throughout neurogenesis, peaking as neurogenesis neared completion.[58] The microglia not only phagocytosed dead cells and cell processes, but also selected living cells, such as progenitors and even mature neurons, for destruction. This sculpting of the brain is carefully regulated, designed to produce the most functional neural and glial architecture.

Disturbance of microglial activation by immune stimulation during a critical stage can adversely affect synaptogenesis.[21] Pruning by microglia is activity dependent, which suggest that excitotoxic stimulation during this period could also adversely affect brain architecture, such as molding of cortical lamination and cortical minicolumns. Bilbo and Schwarz have shown that activation of brain microglia early in life can have long-term consequences on brain function even into adulthood.[20]

## NEURODEVELOPMENT AND MICROGLIA

Early in brain development a special glial scaffolding network derived from astrocytic cells, is formed called radial glial cells.[109] Progenitor cells, which will form both glia and neurons, migrate along this radial glial network toward the pial surface, eventually to form a multilayer cortex. Microglia play a major role in the migration of these progenitors.

### Development and distribution of microglia in central nervous system

Microglia are derived from yolk sac progenitors that seed the brain around 4.5 weeks of gestation in the human brain and appear only when blood circulation develops.[170] Subsequent studies strongly support a mesodermal origin that corresponds to a selective macrophage population (myeloid precursors).[127] Microglia possess specific antigen expressing profiles, proliferative potentials, morphology subtypes, and functions not present in macrophages.[38] Microglia enter the brain via the meninges and choroid plexus. After arriving in the central nervous system (CNS), these microglial progenitor cells then migrate from the germinal zones to the functional layers of the brain. Migration cues include cytokines and chemokines as well as developmental morphogens, growth factors, and glutamate released from the microglia. The microglia use axons, radial glial cell processes, and perivascular sheaths as migratory scaffolds.

While amoeboid microglia migrate along radial glial cells and white matter in most mammals, in humans, microglia also invade and attach to blood vessels within the cortical parenchyma.[200] Once within the parenchyma, microglia become highly proliferative, especially within the white matter.[112] From the second trimester, in humans, microglia become widely distributed throughout the developing brain, mainly in a ramified form, in which there is a significant downregulation of surface immune receptors.[175] Even so, these microglia are fully capable of initiating full immune activation equal to that of the adult CNS microglia, for example, as when challenged by LPS. Interestingly, in fetal microglia, one sees an exaggerated release of glutamate and other excitatory molecules upon immune stimulation that is beyond that seen in the adult. Later during gestation, microglia assume an amoeboid morphology.[200,208,210]

### Development of six-layered cortex, germinal zones, migration of progenitors to pial surface, and emergence of astrocytes and oligodendroglialcytes

The major germinal zones in the brain during embryogenesis include the ventricular zone (VZ) and the subventricular zone (SVZ) [Figure 1]. Tong and Vidyadaran outlined the major events occurring in the developing mouse cerebral cortex.[258] This included proliferation of neuroepithelial cells, which is integral in forming the multilayered neural plate with the innermost apical layer making up the VZ. The proliferation and differentiation continues with the formation of a second germinal zone,

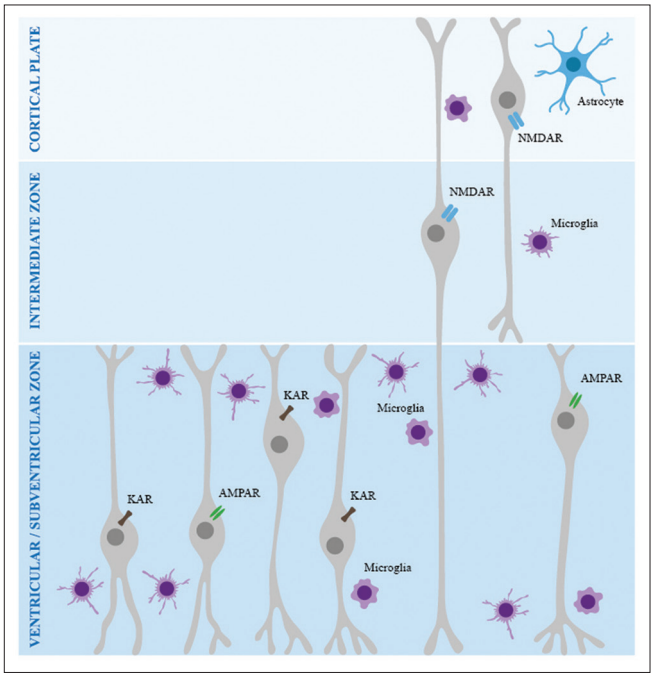


**Figure 1: Microglia and Neurodevelopment. This illustration demonstrates how microglia plays a major role in all stages of neurodevelopment, by a programed release of cytokines and chemokines, a programmed release of glutamate and by active phagocytosis of excessive synaptic units, neurites and even whole cells. Acting at each of the germinal zones, VZ, IZ and CP, microglia controls cell proliferation, migration, angiogenesis, synaptogenesis, and dendritic and axonal development**

the SVZ. During this period, one sees migration of progenitors toward the pial surface and eventual formation of a six-layered cortex. And finally, we see an emergence of astrocytes and oligodendrocytes during late embryogenesis, which occurs just prior to birth.

## Normal microglial "pruning" of neuropil, differences in microglial distribution by sex, and reason for early appearance in males of autism spectrum disorder

Cunningham *et al*. demonstrated that the size of the neuronal precursor pool was regulated by microglia, principally by phagocytosis.[58] It has been shown that with maternal immune stimulation one sees a reduction in the size of the neuronal precursor pool, again by microglial phagocytosis.[49] Population of the developing brain with microglia varies with sex, with males having a significantly greater number of microglia early after birth (P4—postnatal day) than females in brain regions concerned with cognition, learning, and memory (hippocampus, parietal lobe, and amygdala).[214,215] The onset of the dramatic increase in brain microglia in males coincides with the rise in their testosterone levels, somewhere around E18 (embryonic day 18). As a result, males are more likely to be diagnosed with early onset neurological disorders, such as ASD, dyslexia, and ADHD.[21]

Females were shown to have a greater number of microglia than males, but this appeared later in development (P30–60). Interestingly, they also found that most microglia in the P4 males were of an activated (amoeboid) morphology, whereas the females at P30–60 were more often ramified [Figure 2]. Bilbo also found that expression of numerous cytokines and chemokines, as well as their receptors, changed dramatically during development of the rat brain and were highly sex dependent as well.[215] In humans, females increase the number of their microglia with increasing age.[214,215]

## Microglial population of different brain areas

Microglia colonizes different areas of the brain at significantly different rates.[189] For example, in the rat, the hippocampus, amygdala, and cortex are the first to be populated by glia. The striatum is populated by microglia much later postnatally in the rodent brain. Of interest, is the observation that environmental stress accelerates colonization of microglia into the postnatal brain.[96] It has also been observed that changes in the timing of the switch from amoeboid to ramified microglia [Figure 2] can have a profound effect on neural development. This is because of the important part played by amoeboid microglia in synaptic pruning and phagocytosis of apoptotic neurons.

The neuroepithelial cells are the most primitive neural precursors, which give rise to all neurons, astrocytes, and microglia in the CNS. It is from the neural plate that we see the appearance of radial glia, which share properties with astrocytes. Importantly, these cells have specific glutamate transporters.[158] These cells are common progenitors for neurons, astrocytes, and oligodendrocytes. In mice from E16 (embryonic day 16) until birth, there occurs a switch from neurogenesis to gliogenesis, where radial glia stop producing neurons and begin generating astrocytes and oligodendrocytes.[195] At this stage, radial glia are diminished in most areas of the brain, except the cerebellum and retina.

New microglia are not derived from entering macrophages/monocytes, but rather are produced within the brain itself.[37] In the newborn, these microglial are highly proliferative. From E12 onward, increasing numbers

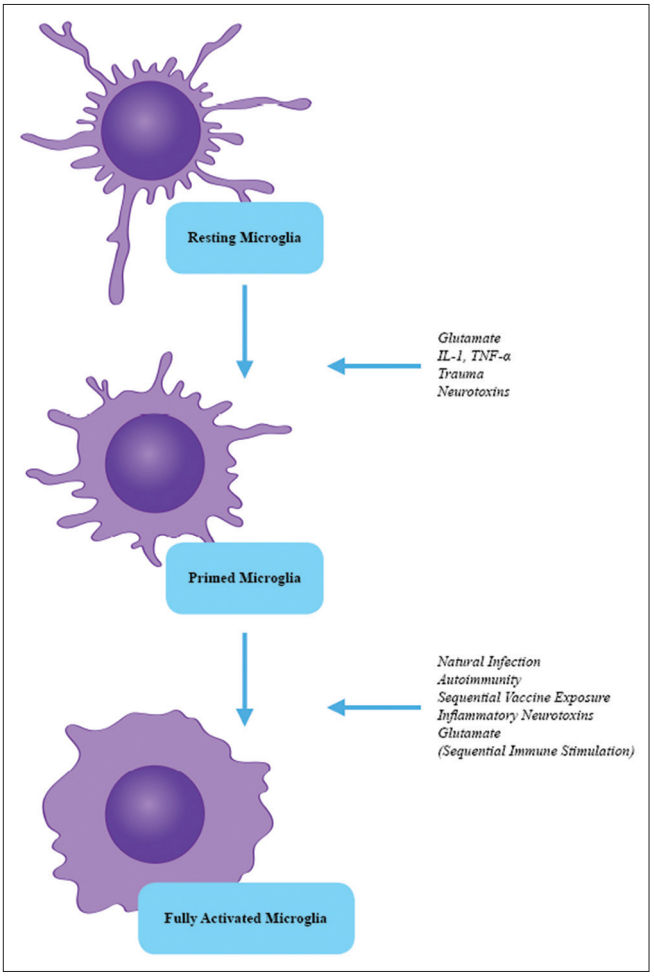


**Figure 2: Microglial Priming and Full Activation by Sequential Immune Stimulation. Demonstrating the transition from a resting (ramified) microglial phenotype to a primed phenotype by an initial immune stimulus. Primed microglia have enhancement of cytokine generation, but no release of cytokines or excitotoxins. The second stimulus fully activates the microglia, resulting in a hyperintense immunoexcitotoxic reaction**

of microglia are found throughout the developing cortex. The densest population of microglia is seen in the VZ and SVZ at this stage.[234,248] These changes are seen in all primates as well as humans.

## Microglia concentration in cerebellum and autism spectrum disorder

Several studies have shown the cerebellum to be the most involved area of the brain in ASDs.[8,16,123] Vargas *et al*. found extensive damage to the cerebellum in both younger and older autistic cases.[264] As with the cortical areas of the brain, microglia also play a critical role in cerebellar development, which includes neurite outgrowth, synaptic pruning, debris clearance, apoptosis, and axon and dendritic development.[160,186] Likewise, microglia during cerebellar development undergo morphologic alterations, changing from amoeboid to ramified as development proceeds [Figure 2].[21] It is now appreciated that the cerebellum has nonmotor functions that include control of attention, working memory, language, emotional elaboration, reward, and other higher functions.[18,213] The human cerebellum matures postnatally with the greatest acceleration of growth and neural organization during the first two years after birth.[2,39]

## Normal microglial pruning of synaptic connections; Abnormal microglia activation directed by compliment tags on synapses leads to abnormal neural development, and miswiring of brain in autism spectrum disorder; positron emission tomography scanning shows areas of microglia activation in autism spectrum disorder patients

Pruning is essential because during early neurodevelopment more synaptic connections are constructed than are ultimately needed by the mature brain. It has been shown that synaptic pruning is activity dependent, during which time large numbers of synapses are eliminated and the remaining ones are strengthened.[116,122,262] During the third week postnatal they also observed increased cell differentiation, cell proliferation, neurite pruning, and synaptic plasticity. In other brain areas, microglia regulate synapses and axons during the second and third postnatal week.[186] Developmental pruning is dependent on the localization of complement tags on synapses chosen for elimination.[235,236] Phagocytic receptors on the microglia (complement receptor 3-CR3/CD11b, CD18/MAC-1) localizes to synaptically enriched areas. Disruption of this signaling results in a sustained deficit, leading to miswiring of the brain. By a yet unknown mechanism weaker synaptic units are tagged for phagocytic removal.

Abnormal activation of microglia and/or the complement system leads to abnormal neural development, as seen in ASD.[235] Microglia are already within the brain when we begin to see neuronal migration, proliferation, and differentiation, and as a result play a major role in the ultimate establishment of functional neural networks by regulating axonal and dendritic pathfinding. Because microglia play multiple roles in brain development and because the programmed changes in morphology are very critical to successful molding of brain architecture, anything that alters this microglial morphological pattern has the potential of significantly altering ultimate brain architecture and brain maturation. It is known that microglial activation is extensive in ASDs.[187,247,264]

Suzuki *et al*. examined twenty men, aged 18–31 with confirmed autism versus matched controls using microglial activation PET scanning techniques utilizing the [$^{11}$C]R-PK11195 tracer.[247] They found extensive microglial activation in several areas of the brains of autistic patients that was not accompanied by microglial proliferation. Microglial activation was significant in the cerebellum, anterior cingulate cortex, orbitofrontal and midfrontal cortical areas, superior temporal and fusiform cortex, and corpus callosum. The most intense activation was in the cerebellum, which conforms to the findings by Vargas *et al*.[264]

## What activates the microglia?

Microglia can be activated by peripheral autoantibodies, as are frequently seen in autistic patients.[230,274] Elevations in peripheral cytokines and chemokines by other mechanisms, such as exposure to neurotoxic metals, certain pesticides/herbicides, stress, trauma, ischemia/hypoxia, and autoimmune disorders, can also activate brain microglia, principally involving IL-1β and tumor necrosis factor-alpha (TNF-α).[28,172]

## Reelen (a glycoprotein in brain development for neurla migration)

Reelin is a critical glycoprotein in brain development. Secreted from Cajal–Retzius cell in the marginal zone reelin plays an important role in neuronal migration in the prenatal and early postnatal brain.[255] Reelin also affects synapse formation and function in the postnatal and adult brain, and these functions are reflected in the manifestation of behavioral and cognitive deficits in animals with reduced levels of reelin.[60] Functional forms of reelin are generated by cleaving of the 400 kDa and 300 kDa isoforms to a 180 kDa form. Fatemi *et al*. reported that autistics have significantly low reelin levels.[76]

## Maternal infections can lead to abnormal neural development as in autism spectrum disorder; Is the RELN gene a factor?

Human studies have shown that maternal infections can lead to several abnormalities of neurodevelopment, such as aberrant neural migration, decreased dendritic arborization, and abnormal cytoarchitecture in the adult,

possibly related to abnormal reelin levels. Some authors suggested that reelin and *RELN* gene were significantly related with psychiatric disorders including ASD.[46] In 2001, the International Molecular Genetic Study of Autism Consortium (IMGSAC) described a region on chromosome 7q as the peak region for linkage to ASD.[113] Given the role of *RELN* in neurodevelopment and its location at chromosome 7q22, *RELN* quickly emerged as a candidate gene for autism and numerous studies (>15) have investigated the occurrence of ASD risk-associated single nucleotide polymorphism in *RELN*.[138] The studies brought mixed results but like *RELN*, their pathological mechanism remains speculative. Lammert and Howell discussing the possible role of reelin in ASD concluded that *RELN* expression is both spatially and temporally consistent with ASD, which is thought to originate as a neurodevelopmental disorder that persists into postnatal life within brain regions including the neocortex, hippocampus, and cerebellum.[138] Some authors suggest that epigenetic processes may play a role in the regulation of *RELN* in ASD brain.

## CYTOKINES AND CHEMOKINES IN AUTISM SPECTRUM DISORDER AND NEURODEVELOPMENT;AUTISM SPECTRUM DISORDER AND IMMUNE DYSFUNCTIONS AND THE ROLE OF EXCITOTOXICITY IN AUTISM SPECTRUM DISORDER

It becomes evident that several cytokines and chemokines play essential roles in brain development regarding migration of progenitors, differentiation, maturation, dendrite arborization, and synaptogenesis. Significant alterations of these immune mediators during critical periods of brain development can also cause varying degrees of functional brain disruptions as well, resulting in abnormal connectivity and cytoarchitecture. Disturbances of these microglial-derived signaling molecules, such as systemic immune activation, would be expected to alter numerous brain pathways and functional systems.

Studies of autistic patients have confirmed the presence of a number of immune dysfunctions, such as neuroinflammation, brain directed autoantibodies, increased T-cell, NK cells, and macrophage responses.[13,164] One study, involving 20 autistic patients, found impaired production of IFN-γ and IL-2 in CD4+ and CD8+ lymphocytes upon stimulation.[133] Li *et al*. in their study of eight autistic patients compared to eight matched normal controls found significant elevation of proinflammatory cytokines TNF-α, IL-6, and GM-CSF (cerebrospinal fluid), INF-γ and chemokine IL-8 in the brains of the ASD patients and no significant difference in IL-10, IL-4 and IL-5, indicating an inflammatory profile.[144] The greatest increase in the ASD patients in their study was in IL-6 and INF-γ. Importantly, this research indicates that ASD children experience increase reactiveness of their peripheral immune system to immune activation and that they also experience priming and activation of their brain microglia in conjunction with systemic immune activation. The Vargas *et al*. study, in which they examined the brains of autistic individuals up to age 45 years, found continuous extensive microglial activation, especially intense in the cerebellum.[264] Migration of progenitor cells, as well as axons, is critical for building brain cytoarchitecture. Young *et al*. found significantly elevated NFκB, a major transcription factor for TNF-α, in samples of the orbitofrontal cortex of autistic patients.[281] NFκB was significantly enhanced in neurons, astrocytes, and microglia in the specimens, with the greatest increase in astrocytes and microglia. Recent studies have shown that IL-1ß can stimulate migration of neurons and guide axonal growth cone turning, essential for forming tangential connections between neuronal columns.[155] TNF-α has also been shown to play a critical role in brain development, but as with other cytokines, depends on careful control of its concentration [Figure 3]. For example, high levels of TNF-α have been shown to inhibit neurite outgrowth and branching in embryonic hippocampal neurons.[176]

Using cerebellar granules cell cultures of individual populations of neurons, Oldeive and Doherty found that TNF-α had highly specific developmental effects on these neurons, depending on the embryonic stage of development.[182] A growing number of studies have demonstrated abnormal systemic immune responses, including elevations of particular cytokines and the presence of antibrain antibodies in ASD.[88,95,101,187,253] Several studies have similarly demonstrated prolonged priming and activation of brain microglia, lasting even decades in ASDs.[91,139,264]

Together, these observations make a compelling case for a link between sequential systemic immune stimulation and neurobehavioral disorders such as the ASDs. Unfortunately, many studies and discussions have omitted or severely downplayed the role of other microglial components linked to immune destruction, such as excitotoxicity.

## GLUTAMATE'S ROLE IN EXCITOTOXICITY AND AUTISM SPECTRUM DISORDER

In 1957, Lucas and Newhouse observed that mice exposed to high-dose monosodium glutamate (MSG) demonstrated extensive neuronal lesions in the inner layer of retinal neurons.[150] Twelve years later, Olney, after reviewing this original observation, repeated the study and discovered not only similar retinal lesions but also selective brain lesions and delayed death processes that involved intense excitation of neuronal activity.[183]

Olney coined the term excitotoxicity to describe the phenomenon. Since the early discovery of excitotoxicity, researchers have carefully worked out the mechanism of excitotoxicity and discovered a series of GluRs with complex structures and intimate interactions among themselves and other neurotransmitters and immune receptors [Figure 4].

## THE GLUTAMATERGIC RECEPTOR SYSTEM – ITS LINK TO AUTISM SPECTRUM DISORDER AND ROLE IN EXCITOTOXICITY: AN OVERVIEW

Subsequent studies confirmed the status of glutamate as a neurotransmitter system and that it involved a number of specific types of receptors. Over 50% of the brain's neurotransmission is via glutamatergic neurotransmission and 90% of cortical neurons utilize glutamate as a neurotransmitter.[286] Several studies have elucidated an array of GluR types, which are defined by the agonists that activate them. The GluRs are classified as ligand-gated ion channels (ionotropic receptors) and GPCR metabotropic receptors (mGluRs).[261,273]

The ionotropic GluRs are the N-methyl-D-aspartate receptors (NMDARs), amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors (AMPAR), and kainate type receptors (KAR). NMDARs are linked to sodium, potassium, and $Ca^{2+}$ channels. It is thought that the entry of excessive $Ca^{2+}$ into the neuron plays a major role in the excitotoxic process by triggering cascades of destructive cellular signaling. Glutamate affinity is greater with NMDARs than AMPAR/KARs but AMPARs are faster conducting [Figure 3].

### Glutamate receptor: NMDAR

Each of these receptor types is composed of a variable arrangement of subunits, which determines their biophysical and physiological properties. The NMDARs are composed of a tetrad arrangement of subtype receptor units—GluN1, GluN2A-D, and GluN3A and B (NR1, NR2A-D, and NR3A-B by the older classification system). Distribution of these variously composed NMDARs varies with their location in the brain. During development, there are significant changes in the composition of these subunits. In the mammalian brain, functional NMDARs require a GluN1 (NR1) subunit associated with one or more GluN2 (NR2) subunits. Magnesium ($Mg^{2+}$) sites within NMDAR channels regulate receptor function, with $Mg^{2+}$ playing a major role as a voltage-dependent channel blocker. With depolarization, the $Mg^{2+}$ block is relieved, allowing the action potential to proceed. Sensitivity to $Mg^{2+}$ blockade also varies with subunit composition.

### Glutamate receptor: AMPAR

AMPARs are composed of GluA1-4 subunits (GluR1-4 by older nomenclature). The presence of the GluA2 (GluR2) subunit in the compositional structure of the AMPA receptor blocks $Ca^{2+}$ entry into the neuron [Figure 3].[57] AMPAR sensitivity is regulated by the trafficking of AMPARs to the neuronal membrane, either containing GluA2 (GluR2) subunit or lacking the GluA2 subunit. The latter causes the AMPAR to be

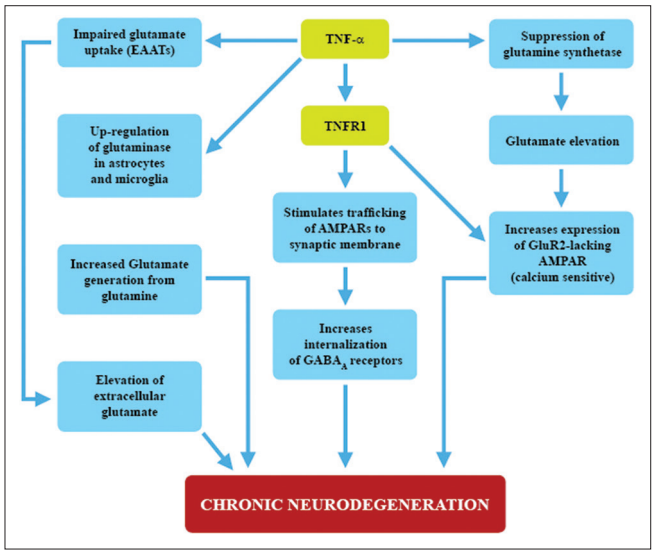


**Figure 3: TNF-Alpha and Immunoexcitotoxicity This chart demonstrates how TNF-α, through one of its receptors (TNFR1) and via oxidative injury, is linked to enhanced excitotoxicity. This involves suppression of GS, trafficking GluA2-lacking AMPARs, upregulation of glutaminase, impaired glutamate transport and endocytosis of GABA receptors.**

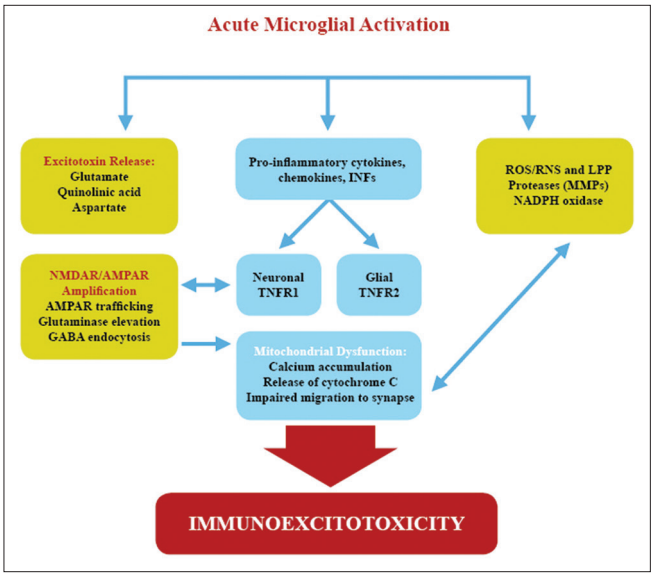


**Figure 4:Aluminum and Aluminofluoride link to excitotoxicity.This diagram demonstrates stimulation of systemic immune activation and microglial activation within the brain by aluminum and fluoride as well as aluminofluoride. $Al^{3+}$, $F^-$ and $AlF_4^-$ all suppresses mitochondrial energy production and trigger ROS/RNS and LPP generation both directly and indirectly via microglial generated inflammatory cytokines, ROS/RNS, LPP and excitotoxicity. This can produce all the core features of ASD**

$Ca^{2+}$ permeable. Trafficking of GluA2-lacking AMPARs to the synaptic unit increases glutamate-induced neuronal activation, as occurs physiologically with LTP (long-term potentiation), plasticity, and during neurodevelopment. GluA2-lacking AMPARs, under pathological conditions, can trigger exaggerated excitotoxic damage, as occurs with a number of neurological conditions, especially with inflammation [Figure 5].[22,78,140]

## Glutamate receptors: Metabotropic receptors (mGluRs)

In addition to the ionotropic GluRs, researchers have identified three major classes of mGluRs with eight subunit types. These receptors operate through G-protein-coupled receptors (GPCRs) via seven transmembrane domains, with an extracellular N-terminal and intracellular COOH terminal domain. Group I mGluRs contain two subtype receptors, mGluR1 and mGluR5, which activate phospholipase C (PLC), producing inositol 1,4,5-trisphosphate, and diacylglycerol (DAG) as second messengers [Figure 5]. This class of mGluRs is principally excitatory, enhancing the excitatory effect of the ionotropic receptors, but under some circumstances can be inhibitory.[71] Groups II and III mGluRs are negatively coupled to adenylyl cyclase and inhibit the excitation of the ionotropic receptors. Group II is composed of mGluR2/3 and Group III is composed of mGluRs 4,6,7,8 subunits.

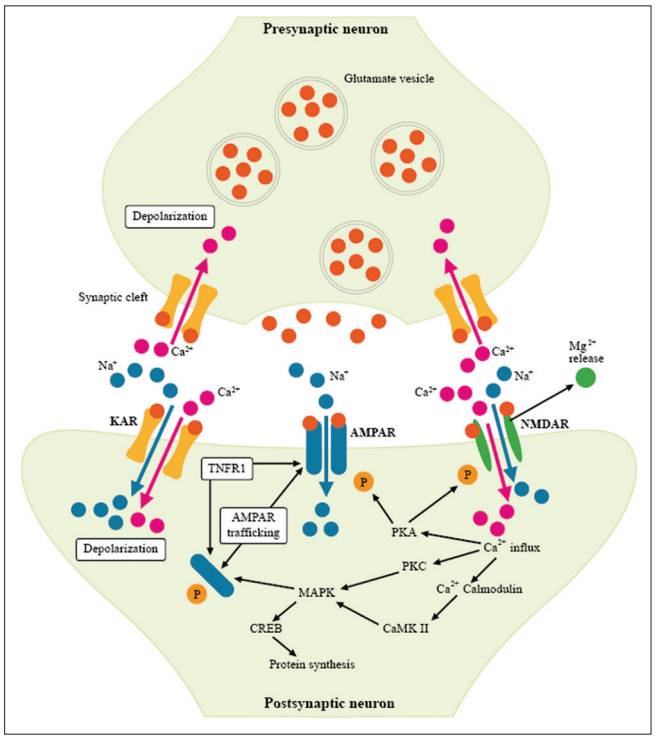


**Figure 5: Synaptic Subunit Trafficking. Illustration of the pre- and post-synaptic units, demonstrating modulation of synaptic strength by trafficking of AMPA and NMDA subunits to the synaptic density. Trafficking of GluA2 (Glu2B)-lacking AMPARs, converts the AMPAR into a calcium permeable receptor, greatly increasing its excitatory strength. Presence of the GluA2 receptor within the AMPAR blocks calcium permeability. Inflammation converts AMPARs to the calcium permeable form**

The mGluRs also vary in location, which affects their physiological effect. Some are close to the postsynaptic density and others occur on the presynaptic axon.[167,174] Group I mGluRs are predominately located postsynaptically where they regulate neuronal excitability. Groups II and III are predominately located presynaptically where they inhibit excitation of neurons. There is a marked variation in the sensitivity of these receptor classes to glutamate. The distribution of mGluRs varies considerably among brain regions and neuron types in the hippocampus.[222] The immense degree of complexity among the various types of GluRs, their heterogeneous distribution, as well as their variable sensitivity to glutamate imparts a high degree of potential variability in functional and pathological reactions to both internal and external environmental conditions. Further complexity and flexibility is imparted by the ability of GluRs to alter their insertion on the synaptic plate by exocytosis/endocytosis, that is, trafficking of receptors and receptor subunits.

## GLUTAMATE AND GLUTAMATE RECEPTORS IN NEURODEVELOPMENT

Glutamate receptors (GluRs) are expressed very early in development and play a vital role in most aspects of neurodevelopment, including brain wiring, synaptogenesis, progenitor migration, differentiation, and maturation of cells.[55] Activation of NMDARs has been shown to activate specific genes controlling neuronal plasticity and neurodevelopment.[91] NMDARs appear to play a major role in neurodevelopment by its control of $Ca^{2+}$ levels, with $Ca^{2+}$ gradients being especially critical for cell migrations.[130] Developmental control by NMDARs is directly influenced by their subunit composition. For example, using mouse cerebellar granules cell lines, Tarnok *et al.* found that increasing NR2B (GluN2B by the new nomenclature) subunit-containing NMDARs increased migration of granules cells *in vivo* and *in vitro*.[252] NR2C (GluN2C), especially in the cerebellum, also played a major role in neuronal migration in this study.

Within the intermediate germinal zone, a site of precursor cell proliferation, and the cortical plate, where neuronal differentiation occurs following radial migration of the precursors, we see further changes with developmental progression [Figure 1]. Within this area, the radially dispersed neurons express glutamate receptors. There is evidence that NMDARs appear in neurons within the cortical plate soon after migrating from the VZ and that these are fully functional receptors (GluRs).[149] In mice, glutamate has been shown to be a powerful chemoattractant for neuronal migration from the VZ/SVZ.[17] Using ferrets, Herrmann

demonstrated that glutamate was present in the VZ, intermediate zone, developing cortical plate, and marginal zone as early as embryonic day 34 (E34), seven day before birth.[106] GluR1 (GluA1- new nomenclature), a subunit of AMPARs, emerged more slowly, with only a few neurons having this subunit in the marginal zone and VZ, but became abundant in the marginal zone and subplate as synaptogenesis begins [Figure 1]. Cells staining for AMPAR subunits increases dramatically during the first two weeks postnatal, reaching maximal levels by the third week. GluR2/3 follow a similar pattern and appear mostly on pyramidal cells.

Furuta and Martin examined sheep neocortex during cortical lamination, specifically for expression of AMPARs (GluR1, GluR2/3 and GluR4), KARs (GluR6/GluR7), and mGluR5.[84] They found that there was distinct, temporal localization of GluRs during neocortical development. They also observed that each GluR had a differential localization within the marginal zone, cortical plate, and subplate. At term, the localizations of the various GluRs changed during cortical plate segregation, which resulted in highly differential distributions within the neocortex at term.

Smith and Thompson, examining the visual cortex of ferrets, found that NMDARs and non-NMDARs follow quite distinct developmental patterns.[233] For instance, NMDARs were low at birth and increased progressively over the first two postnatal months—rising three-fold in layers II/III and nine-fold in layer VI. AMPARs were abundant at birth and their density remained rather constant for the first postnatal month. Their expression reached maximum levels at the period of eye-opening, which occurs on postnatal day 32 in ferrets and soon after birth in humans.

Similar patterns of expression for the various subunits have been confirmed in the developing human brain as well.[202] These studies demonstrate that successful architectonic development of the brain, especially the laminar development of the neocortex, requires a very meticulously controlled pattern of GluR appearance and carefully controlled glutamate levels.

Sodium-dependent glutamate transporters have also been shown to play a major role in neurodevelopment, primarily by controlling glutamate levels, and hence, GluR activation during developmental stages. In humans, there have been five identified glutamate transporters, EAAT1-5. In animal species, GLAST represents EAAT1 and GLT-1, EAAT2. EAAT1 and EAAT2 are present on the cell membranes of microglia, astrocytes, and oligodendrocytes. EAAT1 (GLAST) is the major glutamate transporter in the cerebellum, being mostly expressed in astrocytes.[263] EAAT2 (GLT-1) is the most abundant glutamate transporter in the brain itself, accounting for 90–95% of glutamate uptake in the forebrain. Like EAAT1, it is mostly expressed in astrocytes. While the main function of EAATs is to move extracellular glutamate into glial cells and neurons, under certain conditions one can see reverse transport with movement of glutamate into the extracellular space.[12] This external movement of glutamate from the glia increases the likelihood of excitotoxicity, and in the developing brain, disruption of architectonic development.

Furuta *et al*. found that in the rat, GLAST levels were low prenatally in the forebrain, but were high in Bergmann glia in the cerebellum early postnatally.[85] Levels of GLAST rose in the forebrain later postnatally. EAAT4 was found mainly in the cerebellum, localized to Purkinje cells and levels increased with age. These transporters increased at birth and rose to adult levels between Postnatal day 20 (P20) and Postnatal day 30 (P30).[134]

## GROWTH CONES, GLUTAMATE RECEPTORS, AND CALCIUM GUIDANCE

Growth cones are specialized structures located at the tip of developing axons that act as guidance mechanisms for successful union with synaptic partners. Located at the leading edge of the growth cone are special structures called filopodia and lamellipodia, which guide the axon to its destination among an enormously complex array of possibilities. A number of studies have identified GluRs, in particular NMDARs, within the membrane of growth cones.[69] Once the migration was complete, NMDARs were removed from the growth cone membrane by internal trafficking. Vesicles within axonal growth cones and their filopodia are thought to be able to release glutamate, thus acting though autoreceptors.[209] Glutamate gradients act as chemoguidance signals for circuit construction.

Activation of the growth cone NMDARs produce calcium ($Ca^{2+}$) oscillations that act as guidance signals for axon movement, which includes neuron migration, neurite outgrowth, motility, axon turning, and activation of intracellular signaling pathways involving Rho GTPases, all of which lead to brain architectural construction.[151] There appears to be a permissive range of $Ca^{2+}$ for growth cone function, with low and high levels inhibiting growth cone extension and activity.[121] Glutamate in low concentrations has been shown to inhibit dendritic outgrowth and dendritic pruning, with higher levels resulting in neuron death, most likely by regulating $Ca^{2+}$ levels.[163] The cerebral cortex is composed of a series of diverse cytoarchitecturally distinct columns with extensive interconnecting linkages. How these columns form is poorly understood, but recent research suggests that $Ca^{2+}$ gradients are playing a major role. $Ca^{2+}$ gradients play a critical role in other developmental functions, such as neuron proliferation, dendrite formation and extension, axon guidance, and growth cone function.[198] $Ca^{2+}$ also plays a critical role in ending

neuronal migration, with a reduction in $Ca^{2+}$ transients signaling an ending of migration.[135]

Disruption of cortical column cytoarchitecture and connectivity is characteristic of ASDs. For example, Damarla *et al*. examined high functional autistics using a combination of behavioral testing, functional MRIs, functional connectivity, and corpus callosum morphometric methological tools, and found that autistics had lower functional connectivity between higher order working memory/executive areas and visuospatial regions (between frontal and parietal-occipital regions).[59] Others found functional connectivity defects between the anterior and posterior insula and specific brain regions involved in emotional and sensory processing.[67] A more recent study using DTI imaging described significant abnormalities in white matter development of the cingulum bundle among 21 ASD adolescents compared to 21 age and sex-matched healthy volunteers.[111] They also found lower fractional anisotrophy within the cingulum bundle, which was associated with worse behavioral control.

Underconnectivity, with severe changes in many brain structures, has also been shown for the cerebellum, an area of the brain most affected in autism.[56] These severe changes are not seen in postnatal onset ASD. One explanation for the extreme vulnerability of the cerebellum is because it has a much longer developmental period, especially postnatally, than that in the cerebrum.[254] Because of the prolonged developmental period associated with cerebellar development, injuries by environmental agents, inflammation, and excess glutamate become much more likely.[18] This again demonstrates the critical nature of careful regulation of glutamate gradients within the developing neocortex and that abnormal levels of glutamate during critical stages of neurodevelopment can have serious consequences on brain circuit construction. We also see considerable changes in the levels of various neurotransmitters, such as glutamate, aspartate, GABA, glycine, and taurine during cerebellar development, which are temporally regulated.[168] Disturbances in the programmed rise and fall of these neurotransmitters can alter the architectonic development of the brain, especially the cerebellum.

Synaptogenesis and synaptic pruning within the brain follows a programmed timeline that is specific for individual areas of the brain. A recent study found that synaptic density begins to decline at puberty and is completed during adolescence, within the prefrontal cortex. Substantial elimination of synaptic spines continues beyond adolescence, well into the third decade.[53] Dendritic development begins early, with cortical neurons slowly developing dendrites during the first two trimesters of gestation.[132] The earliest formation of dendrites being within the subplate and deeper cortical layers. Dendritic development accelerates from the third trimester onward and remains highly active until the end of the first postnatal year, making dendritic development vulnerable to radical changes in inflammatory mediators and excitatory neurotransmitters for a considerable period during development. In the human neocortex, dendritic development is most active during infancy and early childhood.[110] $Ca^{2+}$ signaling, mostly regulated by NMDAR activation, has been shown to play a major role in dendritic growth and branching as well as the formation of dendritic spines.[131] Further, NMDAR signaling, which acts at sites of synaptic contact, plays an important role in dendritic spine formation.

## Glutamate receptors and the cerebellum in autism spectrum disorder

Within the cerebellum, it has been shown that GluA2 receptors, an AMPAR subunit that reduces $Ca^{2+}$ influx, is required for normal development of Purkinje cell dendrites.[249] It was also shown that excess $Ca^{2+}$ inhibited dendrite formation and maturation, which would occur with either GluA2-lacking AMPAR insertion and/or overactivity of NMDARs. Increased trafficking of GluA2-lacking AMPARs ($Ca^{2+}$ permeable) occurs with inflammation.[140] In both instances, excess glutamate within the extraneuronal space could disrupt dendrite formation and result in a dysfunctional brain.

## Glutamate receptors and synapse formation

Presynaptic NMDARs also play a major role in synapse formation, and stabilization. GluN2B (NR2B) is essential for neural pathway construction and is widely expressed in the brain during neurodevelopment.[77] Periods of higher NMDAR expression coincides with periods of intense synaptic formation. Excess glutamate stimulation, as occurs during inflammation and other conditions of intense microglial activation, could switch conditions from one of active synaptic development to synaptic damage. ASDs are typically diagnosed before age 3, which is a period of intense synaptic plasticity.[110] During brain development, especially postnatal development, one sees intense synaptic construction, which in the visual cortex of humans peaks during infancy (around 3 months) and at approximately 15 months postnatal for the prefrontal cortex. Synaptic density gradually decreases thereafter so that synaptic density is reduced somewhere around 40% between puberty and adult age in the macaque monkey, which closely resembles that of humans.[35] Synaptic pruning is highly dependent on GluRs and follows a tightly regulated schedule, as described. Microglia appear to be major players in synaptic pruning both as sources of glutamate and by phagocytosis of neurites and whole neurons.[186] Another critical process occurring during CNS development is switching of GluR subunits. It has been shown that early in development, GluN2B subunit is predominant in brain NMDARs, and that as the brain matures postnatally, there is a switching to GluN2A subunits as found in the adult brain.[102] This switching was shown to play a vital role in the synaptic integration of AMPARs.

### Glutamate receptors: Metabotropic GluRs and neurodevelopment

Metabotropic GluRs have also been shown to play a major role in neurodevelopment.[43] For example, group I mGluRs, which include the subunits mGluR1 and mGluR5, both have a fixed temporal developmental pattern. Early after birth, mGluR 1 levels are low and progressively increase with advancing postnatal development. The opposite pattern is seen with mGluR5, which exist in high levels at birth and progressively declines as the brain matures postnatally. The mGluR5 receptors are more often seen in in zones having active neurogenesis. During the first two weeks of postnatal life one sees high concentrations and widespread mGluR5 activity and responsiveness.[148] Interestingly, mGluR5 is the only mGluR subtype located on embryonic neural stem cells. It is also of interest that mGluR1 activation increases the level of reelin mRNA in cultured cerebellar neurons.[162] An important observation as regards ASDs is that inflammation during fetal development has been shown to reduce reelin levels in the developing brain.

### Glutamate receptors and autism spectrum disorder; A short neurobiochemical summary

Together, these studies strongly suggest that glutamate and its receptors are playing a major role in the progressive elaboration of the architecture of the developing brain and that a number of internal and external environmental conditions can alter both glutamate levels and GluR physiology, resulting in varying degrees of abnormal brain development.

## IMMUNOEXCITOTOXICITY IN BRAIN DEVELOPMENT AND AUTISM SPECTRUM DISORDER

A growing number of studies are defining an interrelationship between the immune system and excitatory neurotransmission. This includes alteration of glutamate transporters as well as direction of glutamate transport, trafficking of $Ca^{2+}$-permeable AMPARs and NMDARs, elevation of glutaminase activity, and suppression of mitochondrial function with alteration of mitochondrial energy generation, thus enhancing excitotoxicity [Figure 5].

## FROM ALTERATION OF THE IMMUNE RESPONSE TO GLUTAMATE RECEPTORS TO EXCITOTOXICITY WITH EXCESS GLUTAMATE AS A NEUROTRANSMITTER

One of the principle control systems of extraneuronal levels of glutamate is accomplished by a series of five glutamate transport proteins (EAAT1-5) discussed above. Both inflammatory cytokines and excitotoxins, alter the redox status of EAATs, making them dysfunctional, thus allowing the accumulation of extracellular glutamate and possible excitotoxicity. Extraneuronal glutamate can also be altered by another system called the cystine/glutamate antiporter $Xc^-$. Under normal conditions, this system exchanges extracellular cystine for intracellular glutamate, thus raising extracellular glutamate levels, which are then quickly lowered by EAATs [Figure 6]. When the EAATs are rendered dysfunctional by reactive oxygen/reactive nitrogen species (ROS/RNS), 4-hydroxynoneal and pro-inflammatory cytokines, glutamate levels will rise in the extracellular space and trigger excitotoxicity over a prolonged period.

## REPEATED IMMUNE ACTIVATION LEADS TO MICROGLIAL ACTIVATION, EXCESS GLUTAMATE, AND NEUROPATHOLOGICAL CHANGES IN THE NEUROPIL THAT CAN BE BLOCKED BY GLUTAMATE RECEPTOR BLOCKERS

Prolonged and repeated systemic immune activation appears to be central to ASD etiopathology. With subsequent, especially closely spaced immune stimulation, full activation of the overactive primed microglia occurs, leading to progressive pathological changes in the developing brain. This is especially so if the episodes of systemic immune stimulation are closely spaced together.[26] It has been shown that inflammatory cytokines, such as TNF-α, do not cause neuronal destruction by itself, but rather enhance glutamate excitotoxicity [Figure 3]. For example, LPS- or TNF-α stimulates macrophages and microglia to induce robust neurotoxicity, but the destruction was completely blocked by MK-801 (dizoclipine), an NMDAR blocker.[279] Further, they demonstrated that blocking glutaminase, the enzyme that converts glutamine into glutamate [Figure 7], also blocked TNF-α and LPS neurotoxicity, as did blocking gap junctions, a principle exchange system for glutamate release. Bal-Price and Brown demonstrated similar

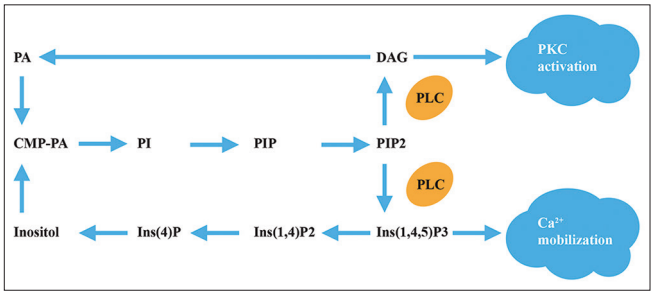


**Figure 6: A simplified scheme of the phosphoinositide signaling system. Phosphatidylinositol 4,5-bisphosphate (PIP2) in the plasma membrane is hydrolyzed by phospholipase C (PLC) after GPCR activation and yields inositol 1,4,5-trisphosphate (Ins(1,4,5) P3) and diacylglycerol (DAG). Both products of this hydrolysis have a second messenger role. Ins(1,4,5)P3 binds to a receptor in membranes of endoplasmic reticulum, which results in a release of $Ca^{2+}$ into the cytosol. DAG activates protein kinase C (PKC). The coupling between the GPCR and PLC is mediated by G proteins. PA – phosphatidic acid, PI – phosphatidylinositol.**

finding using astrocytes and microglia exposed to LPS or interferon-γ in which blocking nitric oxide (NO) production induced by the inflammatory cytokines, prevented neurotoxicity.[14] Further, they found that the NO rapidly inhibited mitochondrial respiration and this increased glutamate release from neuronal/astrocytic cultures. Blocking the NMDARs using MK-801 also prevented neuronal death in these same cultures.

## INTEGRATION OF IMMUNE DYSFUNCTION, GLUTAMATE RECEPTOR CHANGES, MICROGLIAL ACTIVATION, AND DISRUPTION OF NEURODEVELOPMENTAL MILESTONES LEADING TO PATHOLOGIC CHANGES IN AUTISM SPECTRUM DISORDER

In most studies, the target was neurodegeneration, yet the effects of elevation of glutamate brain levels during neurodevelopment can occur at concentrations lower than needed to cause neurodegeneration. This means that microglial activation alone, as long as it is associated with an elevation of glutamate levels, holds the possibility of disrupting neurodevelopmental milestones, particularly if occurring at periods when glutamate levels should fall. This would especially be of concern should inflammation be prolonged and associated glutamate levels were constantly elevated as well, as we see in ASDs.[264] Inflammation has also been shown to enhance excitotoxicity through both NMDA and AMPARs. IL-1ß, for example, has been shown to enhance the sensitivity of NMDARs by several mechanisms, including microglial recruitment and activation, and by stimulating NMDAR trafficking to the synaptic membrane.[157,269] It has also been shown that TNFα and glutamate, each in concentrations too low to initiate neurotoxicity, when combined, produce robust

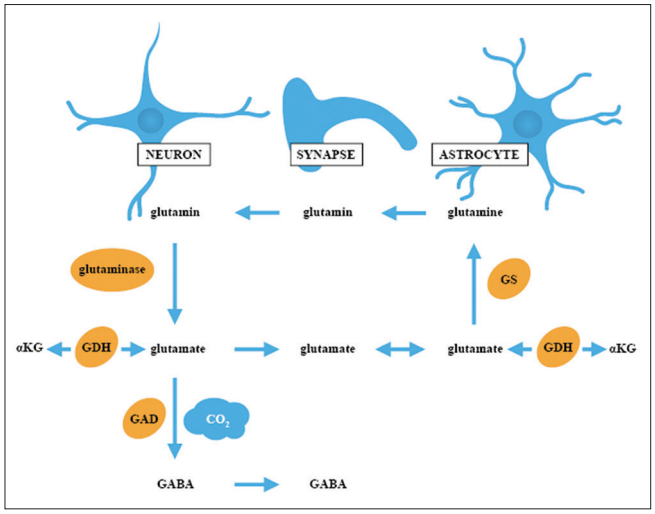


**Figure 7: At each of these levels we see enzymatic conversion of glutamine into glutamate with protective systems to prevent excessive accumulation of glutamate by entry into kreb's cycle and by conversion into GABA**

neurotoxicity.[79] TNF-α increased glutamate-induced neurotoxicity by a number of mechanisms, such as microglial recruitment, upregulating glutaminase, inhibition of glutamine synthetase, inhibition of EAATs and stimulation of TNFR1, which increases trafficking of GluA2-lacking AMPARs [Figure 3].[143,180]

Of particular interest is the inflammation-induced enhanced trafficking of GluRs and alteration of glutamate subunits, as well as inflammatory cytokine-stimulated endocytosis of GABA receptors, which would shift the balance in the brain toward excitotoxicity. The interaction between immune mediators and GluRs triggers a neurotoxic reaction, which involves the generation of high levels of ROS and RNS, along with LPP. These neurotoxic compounds have been shown to inhibit glutamate transport proteins (EAATs), inhibit metabolic glutamate-clearing enzymes, such as glutamine synthetase (GS), glutamate dehydrogenase (GDH) and glutamic acid dehydrogenase (GAD), as well as interfere with mitochondrial energy generation [Figure 8].

Interestingly, maternal inflammation has also been associated with a significant increase in glutaminase, an increase in NMDAR subunit GluN2 (NR2) expression and impairment of GLT-1 function, all things that trigger excitotoxicity.[284] Because of the interaction between GluRs and their subunits and inflammatory mediators, it is difficult to separate which component of immunoexcitotoxicity is actually playing the most important part in altering neural development and laminar architecture of the cortex. Most papers written on this subject have concentrated on abnormalities induced by excess immune stimulation on architectonic development and this is especially so with studies on ASDs. Yet, the two mechanisms seem to be inextricably linked. As an example, a recent paper by Rossignol and Frye reviewed the literature on inflammation and its relationship to autism but never mentioned excitotoxicity or GluRs dysfunction.[206] Most of the autism literature tends to follow this pattern.[24,41,108,164] Yet, a growing literature strongly suggest a link to abnormalities in glutamate and/or GluR function as major players in ASDs, which includes children, high functioning autism, and adults with autism.[80,223,224,282]

The ability of immune cytokines and other immune mediators of inflammation, to enhance microglial priming/activation and to magnify the excitotoxic potential of GluRs, indicate that the two processes operate together, both physiologically and pathologically.

## THE ROLE OF FLUORIDE AND ALUMINUM IN ETIOPATHOLOGY OF AUTISM SPECTRUM DISORDER

A considerable amount of scientific evidence demonstrates that fluoride and $Al^{3+}$ can harm a great

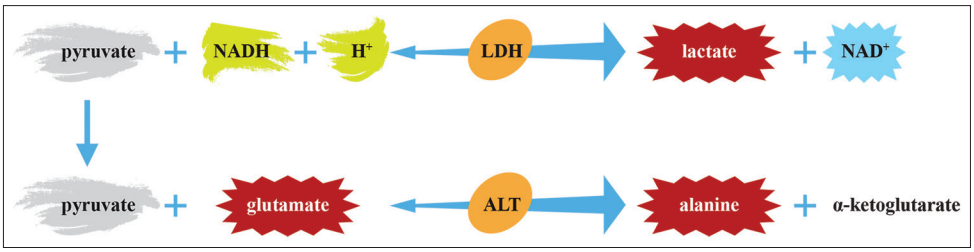


**Figure 8: Peripheral markers of disturbances of mitochondrial energy metabolism in ASD. Patients with ASD have raised blood lactate, alanine, and glutamate. LDH – lactate dehydrogenase; ALT – alanine transaminase**

number of physiological functions, including critical regulatory enzymes, mitochondrial energy production, neurotransmitter and endocrine signaling, brain development, higher CNS functions, and immune system dysfunctions.[42,240]

## FLUORIDE AS A NEUROTOXIN IS IN ENVIRONMENTAL EXCESS IN GROWING YOUNG CHILDREN

Fluoride is a ubiquitous compound found in drinking water (both naturally and added) in many soils, incorporated within edible plant components, and is considered a natural compound. Millions of people live in endemic areas with high concentrations of fluoride in groundwater and in the biosphere. Fluoride exposure is common in fetuses, newborns, and small children. The United States Environmental Protection Agency (EPA) has done both a dose-response analysis and a relative source contribution analysis.[72] This data show that at the 90$^{th}$ percentile a third of children between the ages of 6 months and 4 years are getting significantly more fluoride than is considered safe. For infants up to 6 months old receiving infant formula, if the local drinking water fluoride level is higher than 0.8 mg/L, the intake of fluoride will exceed 0.1 mg/kg/day, and this level is 100 times higher than the level found in breast milk (less than 0.001 mg/kg). Many Asian and Latin American countries have reported concentrations of fluoride often exceeding the WHO guideline values of 1.5 mg/L (1 ppm).[72,240]

## EXCESS FLUORIDE AND NEURODEVELOPMENTAL DEFECTS

Fluoride is not an essential element for human growth and development. Prolonged exposure to fluoride in the prenatal and postnatal stages of development might have toxic effects on the development and metabolism of brain. In fluoridated areas, we observe some core symptoms of ASD in some individuals. These include IQ deficits, hypocalcemia, hypomagnesemia, hypothyroidism, sleep-pattern disturbance, inflammation, impaired ability of cognition, learning, and behavioral problems. Blaylock was the first to suggest that excitotoxicity may be a central mechanism of fluoride toxicity.[27] Despite that epidemiological studies have documented fluoride among developmental neurotoxicants, fluoride is not included among the environmental factors influencing the risk of autism.[97,177,206,280] Extensive research provides evidence that fluoride affects life processes from fertilization to ageing, from gene transcription to cognition.[240] In addition to the interpretation of laboratory investigations using isolated cells/tissues or animal models, many epidemiological, ecological, and clinical studies have shown negative effects of fluoride on domestic and laboratory animals and humans.[237,240]

## ALUMINUM AS A NERUOTOXIN

There are a number of $Al^{3+}$ sources, such as the drinking water, dietary substances, cosmetics, and the widespread use of $Al^{3+}$ in medicine, namely in vaccines. Many investigations show that $Al^{3+}$ can elicit impairment of development and immunity; that it acts as a hormonal disruptor, a neurotoxin, and elicits intense and prolonged activation of brain inflammation. $Al^{3+}$ toxicity in humans, especially as regards the CNS, has been studied and discussed by several authors [e.g. 23,171,256,271]. It is not surprising that $Al^{3+}$ appears among the environmental toxins, which can participate in the etio-pathology of ASD.[4,193,239]

## INCREASE IN EXPOSURE TO ALUMINUM; RELATIONSHIP BETWEEN ALUMINUM EXPOSURE AND INCREASE IN AUTISM SPECTRUM DISORDER

Among the suspected environmental toxins surveyed, $Al^{3+}$ and $Al^{3+}$-adjuvants have increasingly correlated positively to the rise in ASD.[172] The trend in $Al^{3+}$-adjuvants exposure is also notable in that very young infants have experienced the largest relative increase in numbers of vaccination from the early 1980s to 2005. In an extensive review, Tomljenovic and Shaw pointed out that 18 $Al^{3+}$-containing adjuvanted vaccines are included in the current pediatric vaccine schedule.[256] They found that (1) children from countries

with the highest ASD incidence appear to have the highest exposure to $Al^{3+}$ from vaccines; (2) the increase in exposure to $Al^{3+}$- adjuvants significantly correlates with increase ASD in the United States over the last two decades; and (3) a significant correlation exist between the amounts of $Al^{3+}$ administered to preschool children and the current prevalence of ASD in seven Western countries, particularly at 3-4 months of age. The data satisfied Hill's criteria for causation.

Widespread mobilization of $Al^{3+}$ into our biosphere may place all of humankind at a heightened inflammatory status thereby increasing general risk for the development of ASD and other progressive neurodegenerative disorders associated with an inflammatory component.[220,221] Evidence of the neurotoxicity of $Al^{3+}$ includes the involvement of $Al^{3+}$ in the development of Alzheimer disease and also $Al^{3+}$-adjuvants involvement in the development of autoimmune/inflammatory syndrome induced by adjuvants (ASIA).[24,139]

## ALUMINUM AND FLUORIDE EXPOSURE INDUCES MICROGLIA ACTIVATION, INCREASES GLUTAMATE BLOOD AND BRAIN LEVELS AND ABNORMALITY IN THE DEVELOPING BRAIN AND NEURODEGENERATION

Recent studies have also shown that both fluoride and $Al^{3+}$, as well as $AlF_x$ can induced microglial, astrocyte and B-cell activation, with resulting increases in blood and brain ROS, RNS, and LPP.[229,278] Because both $Al^{3+}$ and fluoride accumulate in the brain with chronic exposure, even low dose exposure of these two elements can eventually result in neurotoxic concentrations. There is compelling evidence from a multitude of studies indicating that fluoride and micromolar $Al^{3+}$.

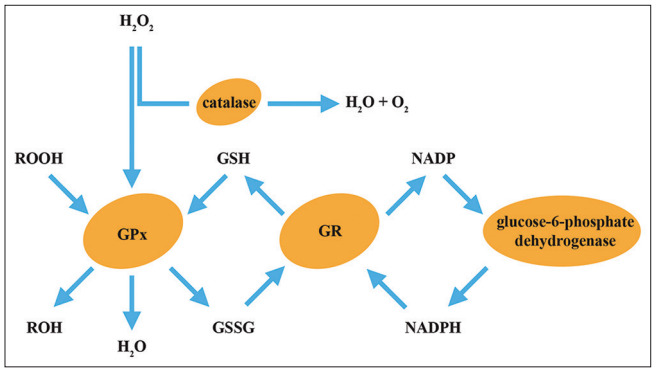


**Figure 9: The GSH redox system. Hydrogen peroxide ($H_2O_2$), the immediate precursor of hydroxyl radical, is metabolized via the action of catalase and glutathione peroxidase (GPx). GPx also metabolizes reactive hydroperoxides (ROOH) and oxidizes reduced glutathione (GSH) to its disulfide form (GSSH), which is recycled back to GSH by the action of glutathione reductase (GR).A cofactor for GR is NADPH, which is supplied by the action of glucose-6-phosphate dehydrogenase**

acting synergistically, can elevate blood and brain glutamate to levels known to cause alterations in the developing brain, as well as initiate brain inflammation, and neurodegeneration. We suggest that both of these ubiquitous environmental toxins have a substantial role in the immunoexcitotoxicity and the etiopathology of ASD.

## METABOLIC AND DEVELOPMENTAL EFFECTS OF FLUORIDE

The highly electronegative fluoride ion with the same size and the same valence orbital as oxygen became a useful laboratory tool in our understanding of the biochemical and biophysical mechanisms of enzyme catalysis underlying biological processes such as metabolism and signal transduction.[93,197,204,237] The toxic action of fluoride has been attributed to the fact that it acts as an enzymatic poison, inhibiting activities of many important enzymes such as enolase, lipase, phosphofructokinase, pyruvate kinase, glycogen synthase, succinate dehydrogenase, cytochrome oxidase, lactate dehydrogenase, glycogen phosphorylase, various phosphatases, ATP-ases, urease, and cholinesterases to name a few. Fluoride is responsible for numerous metabolic disorders and results in a decrease in energy metabolism, redox potential of the cells, and an increase in oxidative stress.[62,285]

Almost 50% of children with ASD may display peripheral markers of disturbances of mitochondrial energy metabolism.[81,206,207] Mitochondrial disease is a heterogeneous group of disorders characterized by impaired energy production due to phosphorylation dysfunction. Lombard (1998) published a hypothesis suggesting an etiological possibility for autism involving mitochondrial dysfunction with concomitant defects in neuronal oxidative phosphorylation within the CNS.[147] This hypothesis was supported by a frequent association of lactic acidosis and carnitine deficiency in autistic patients. Several authors confirmed the high frequency of hyperlactacidemia and increased lactate/pyruvate ratio as well as elevated alanine levels in blood and serum of ASD patients [Figure 8].[240]

Several researchers have reported evidence of mitochondrial dysfunction in ASD brain samples compared to controls.[9,45,94,250] If such dysfunction is present at the time of infections and immunizations in young children, the added oxidative stress from immune activation on cellular energy metabolism is likely to be especially critical for the CNS, which is highly dependent on mitochondrial function. Young children who have dysfunctional cellular energy metabolism, therefore, might be more prone to undergo autistic regression between 4 and 30 months of age if they also have infections or immunizations at the same time.

Recently, Delhey *et al*. measured mitochondrial enzyme activity on the one of the largest cohorts of individuals with ASD studied to date with concurrent measurement of symptoms in a subset and found that children with ASD demonstrated significantly greater variation in mitochondrial activity compared to controls.[63] This study demonstrates, for the first time, that such metabolic variations are related to ASD symptoms.

It has also been demonstrated that suppression of energy generation significantly increases sensitivity to excitotoxicity.[104] Excitotoxicity, as well as inflammation, stimulates the generation of NO by microglia and astrocytes, which directly suppresses mitochondrial function. The mitochondria also act as $Ca^{2+}$ buffering systems, which when disturbed can not only increase excitotoxic sensitivity but also, by altering essential $Ca^{2+}$ waves, will alter neuronal and glial migration.[237]

One of the best documented biochemical changes in ASD is a decrease in cellular glutathione (GSH), a major intracellular antioxidant, and an increase in oxidized glutathione (GSSG), resulting in a reduction in the ratio of reduced (active) GSH to inactive GSSG [Figure 9]. The GSH redox system is important for reducing oxidative stress and thus protecting cellular components. Oxidative stress, defined as an imbalance between oxidants and antioxidants in favor of the oxidants, represents a link between genetic, immunological, and environmental factors underlying ASD.[159]

GSH, a radical scavenger, is converted to GSSG through glutathione peroxidase (GPx), and converted back to GSH by glutathione reductase (GR). GSH can detoxify hydrogen peroxide ($H_2O_2$), preventing the formation of hydroxyl radicals [Figure 10]. All these enzymes are stimulated by melatonin, which also increases GSH production by stimulating its synthesis. Variations in GSH-related pathways have been reported in autistic patients and correlated with their behaviors.[206,240]

The reduced/oxidized GSH-redox equilibrium regulates a pleiotropic range of functions that includes ROS/RNS scavenging, detoxification, maintaining cell membrane integrity, signal transduction, and apoptosis. Under normal physiologic conditions, glutathione reductase activity is sufficient to maintain the high GSH/GSSG ratio. Excessive intracellular oxidative stress could result in GSSG export to the plasma. Therefore, an increase in plasma GSSH is a strong indicator of intracellular oxidative stress, which may have functional consequences such as increased mitochondrial superoxide production and a chronic inflammatory response.[287]

GSH and GSSG levels are significantly changed in autistic individuals, mainly as the consequence of disturbances in processes of transmethylation/transsulfuration. Plasma concentrations of methionine, S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH), adenosine, homocysteine, cystathionine, cysteine, GSH and GSSG have been repeatedly measured in children with autism and found to be disturbed[87,114] [Table 1].

Highly significant ($P \leq 0.001$) decrease of SAM and SAH was also observed in subjects with ASD.[114] These observations were supported by detailed analysis of metabolic and nutritional status of 55 children with ASD ages 5–16 years compared with nonsibling, neurotypical controls ($n = 44$).[3]

Fluoride can contribute to disturbances in GSH homeostasis because it enters the cascade and attaches to the SAH. Fluoride has been identified as an inhibitor of SAH hydrolase.[165] Several reports have indicated that fluoride exposure can reduce the cellular level of GSH and induce oxidative stress in liver, kidney, heart, spleen, and brain.[5,15,19,225,227]

Oxidative stress, which can stimulate microglial activation and immunoexcitotoxicity, may lead to neurodevelopmental abnormalities in children by affecting numerous cellular processes, especially via cell signaling and mitochondrial dysfunction.

## THE EFFECTS OF FLUORIDE ON THE BRAIN AND NEURODEVELOPMENT

Numerous animal studies have been published, which have raised a level of concern about the impact of

**Table 1: Changes in metabolites of methionine-homocysteine cycle in ASD children**

| μmol/L | ASD children Geier *et al.* (*n*) | Healthy controls Geier *et al.* (*n*) | ASD children James *et al.* *n*=80 | Healthy controls James *et al.* *n*=73 | Reference values for children and youth |
|---|---|---|---|---|---|
| Methionine (μmol/L) | 17.6 (28) | 31.5 | 20.6±5.2 | 28.0±6.5 | 28 |
| GSH (μmol/L) | 3.1±0.53 (38) | 4.2±0.72 (120) | 1.4±0.5 | 2.2±0.9 | 4.2±0.74 |
| GSSG (nmol/L) | 0.46±0.16 (38) | 0.35±0.05 (120) | 0.4±0.2 | 0.24±0.1 | 0.2±0.26 |
| Homocysteine (μmol/L) | 5.87 (12) | 9.46 (120) | 5.7±1.2 | 6.0±1.3 | 4.3-10 |
| Taurine (μmol/L) | 48.6±14.0 (38) | 97.5±8.8 (27) | - | - | 95±9 |
| Cysteine (μmol/L) | 17.8±9.5 (38) | 23.2±4.2 (64) | 165±14 | 207±22 | 238±22 |

Geier *et al.*[186] measured the free plasma cysteine, while James *et al.*[186] probably used the value for total cysteine. However, the both values indicate the decrease of cysteine in ASD subjects

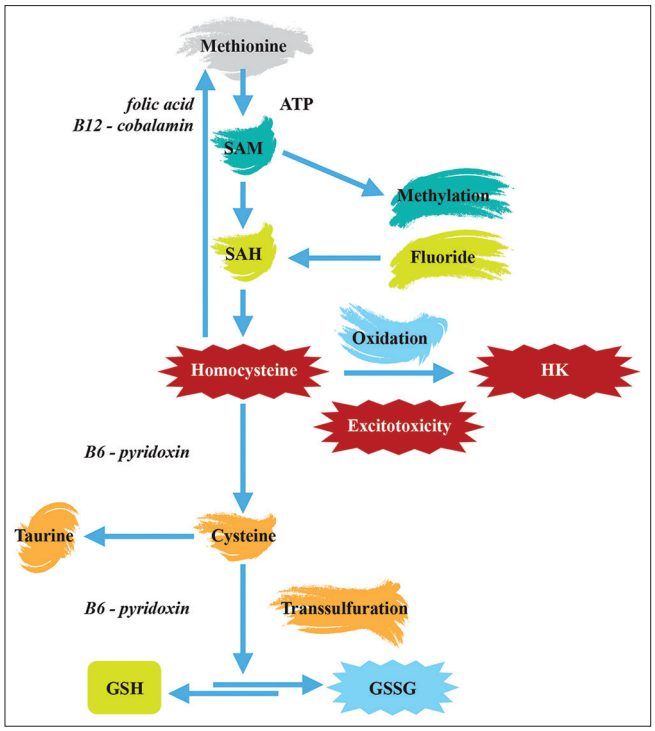


**Figure 10: The simplified scheme of the processes of transmethylation/transsulfuration metabolism. SAM – adenosylmethionine, SAH – S-adenosylhomocysteine, GSH – reduced form of glutathione, GSSG – oxidized disulfide form**

increasing fluoride exposure on the brain of individuals with autism.[19,225,227] Metabolic and mitochondrial defects caused by fluoride may have toxic effects on brain cells, causing neuronal loss and altered modulation of neurotransmission systems. We have shown that mitochondrial dysfunction, oxidative stress, inflammation, and excitotoxicity are key players in the pathogenesis of ASD.[240] Fluoride induces oxidative stress through depletion in the levels of GSH and superoxide dismutase (SOD) with increased levels of lipid peroxidation products (LPP) in the developing brain of rats.[15] In this study, pregnant Wistar rats were dosed with sodium fluoride (20 mg/L) from day one of pregnancy until the pups were aged day 30. Chronic exposure to fluoride resulted in severe behavioral and cognitive impairments. Recently, Adedara *et al.* reported that fluoride from drinking water (15 mg/L) induced oxidative stress markers and decreased GSH levels in the brain of the male rats.[5]

## Human studies validating animal observations of fluoride toxicity

Two studies provided evidence that peripheral markers of oxidative stress observed in plasma and immune cells of subjects with ASD are similarly changed in several brain regions. Chauhan *et al.* found that GSH levels were significantly decreased by 34.2 and 44.6%, with a concomitant increase in the levels of GSSG by 38.2 and 45.5%, respectively, in the cerebellum and temporal cortex from patients with autism compared to the control group.[44] There was also a significant decrease in the levels of total GSH by 32.9% in the cerebellum, and by 43.1% in the temporal cortex of patients with autism. The redox ratio of GSH to GSSG was also significantly decreased by 52.8% in the cerebellum and by 60.8% in the temporal cortex of patients with autism, suggesting GSH redox imbalance in the brains of individuals with autism.

Rose *et al.* examined frozen samples from the cerebellum ($n$ = 15) and Brodmann area 22 (BA-22) of the temporal cortex ($n$ = 12) from individuals with autism and unaffected controls. GSH and GSH/GSSG were significantly decreased in both the autistic cerebellum and BA-22.[205]

However, unlike systemic cells, brain neurons depend on the transfer of astrocytic generated GSH, which is produced by the glutamate/cystine antiporter $X_c$-. The glutamate/cystine antiporter $X_c$- is an exchange system involving an exchange of intracellular glutamate for extracellular cystine. Intracellular cystine is broken down into cysteine, which is metabolized to GSH. Cysteine is the rate-limiting substrate for GSH and, along with cystine, it also forms a key redox couple on its own. That is, they act to reduce oxidative stress. High levels of extraneuronal glutamate inhibit the exchange and can lower brain GSH.[142] This increases the vulnerability of the brain to oxidative stress and immunoexcitotoxicity.

## Human studies on impaired neural development found in brains of aborted fetuses in an endemic flourosis area of China by a pathological study and by a IQ assessment study

Based on the research from China, the fetal brain is highly susceptible to fluoride poisoning.[50,51] The fetal blood–brain–barrier (BBB) is immature and readily permeable to fluoride. Elevated fluoride content was found in embryonic brain tissue obtained from government required abortions in areas where fluorosis was prevalent. A study by Du revealed adverse effects of fluoride on the brains of 15 aborted fetuses between the 5–8$^{th}$ months of gestation from an endemic fluorosis area in China compared with those from a non-endemic area.[66] These studies showed poor differentiation of brain nerve cells and delayed brain development. One may conclude from these studies of chronic fluoride overload during the course of human intrauterine fetal life that fluoride may produce certain harmful effects on the developing brain of the fetus.

## Meta-analyses studies of intelligence in children with high fluoride exposure

Tang *et al.* published a meta-analysis of 16 studies carried out in China between 1998 and 2008 evaluating the influence of fluoride levels on the IQ of children.[251] These authors concluded that children living in an area with a high incidence of fluorosis and high ambient air

fluoride levels have a five times higher odds of developing a low IQ than those who live in a low fluorosis area.

Findings from meta-analyses of 27 studies published over 22 years suggest an inverse association between high fluoride exposure and children's intelligence.[50,51] Children who lived in areas with high fluoride exposure had lower IQ scores than those who lived in low-exposure or control areas. These findings are consistent with an earlier review, but are more comprehensive in (a) including 9 additional studies, (b) performing meta-regression to estimate the contribution of study characteristics as sources of heterogeneity, and (c) estimating pooled risk ratios for the association between fluoride exposure and a low/marginal Raven's test score.[251]

### More human studies on the inverse association of fluoride exposure and IQ

The team of Fluoride Action Network summarized 57 human studies; 50 of which found that elevated fluoride exposure is associated with reduced IQ and only 7 studies found no such association (http://fluoridealert.org/studies/brain01/). The human studies are based on IQ examination of over 12,000 children. Reduction of children's intelligence and various psychiatric symptoms, such as memory impairment, difficulties with concentration and thinking were reported from various geographic regions. Even in studies with methodological limitations, IQ reduction is a consistent conclusion. Many of the fluoride/IQ studies have used relatively simple designs and have failed to adequately control for all factors that can impact a child's intelligence (e.g., parental education, socioeconomic status, lead, and arsenic exposure). For several reasons, however, it is unlikely that these limitations can explain the association between fluoride and IQ. Indeed, the studies that controlled for certain key factors (e.g., arsenic, iodine, etc.) reported some of the largest associations between fluoride and IQ to date. A study from Mexico, which examined 61 children aged 6 to 8 years from a community with 1.2 to 3 ppm of fluoride in their drinking water, found that urinary fluoride correlated positively with reaction time and inversely with the scores in visuospatial organization.[203] The authors concluded that these changes could affect the attention process and the reading and writing abilities in children. Approximately 14 million people live in high-risk areas in Mexico. Significant inverse relationship between the fluoride concentration in drinking water and IQ ($r$ value = -0.204; $P < 0.000$) was reported by several authors in India.[11,125,173] The effect of high water fluoride concentration on the intellectual development of children has been observed in some regions of Iran.[216]

The results from several geographic regions support the view that fluoride may be a developmental neurotoxicant that affects brain development at exposures much below those that can cause toxicity in adults. Serum fluoride concentrations associated with high intakes from drinking water may exceed 1 mg/L (50 μmol/L) – more than 1,000 times the levels of some other neurotoxicants that cause neurodevelopmental damage.[251]

## FLUORIDE AND ENDOCRINE DISRUPTIONS

### Fluoride and thyroid dysfunction

Disturbance of thyroid hormone production has been found in correlation with lowered IQ in children. The investigations from India demonstrated that the thyroid gland appears to be very sensitive tissue in the body in relation to fluoride burden. Susheela *et al*. compared the production of free thyroid hormones (FT3/FT4) and thyroid-stimulating hormone (TSH) of 90 children living in fluoride endemic, non-iodine deficient areas of Delhi, India, along with 21 children from non-endemic areas.[246] The serum fluoride concentration also had a significant relationship with FT3/FT4 and TSH concentrations in school children in an endemic fluoride area of Udaipur district, Rajasthan.[231]

### Thyroid deficiency leads to brain damage in autism spectrum disorder

Thyroid hormones are essential for brain maturation and for brain function throughout life. Thyroid hormone deficiencies even for short periods, may lead to irreversible brain damage, the consequences of which depend on the specific timing of onset and duration of thyroid hormone deficiency.

Thyroid dysfunction is frequently found in children with ASD.[92,105,107,177,232] Significantly reduced levels of TSH in ASD have been observed by Hashimoto and colleagues, where they examined 41 ASD boys (average age of 5.7 years) compared to 5 typically-developing (TD) boys.[105] Reduced levels of TSH were also observed in blood spots from infants who later were found to have ASD ($n = 16$ ASD and $n = 32$ TD); gender not reported), suggesting that TSH levels may be useful as an early biomarker for ASD. TSH levels were also significantly lower in 30 boys with ASD in comparison with and 30 TD typically-developing boys, ages 2–8 years.[232] Recently, Frye *et al*. measured blocking and binding folate receptor autoantibodies (FRAAs) and TSH, FT4, total T3, thyroid-releasing hormone and other metabolites in 87 children with ASD, 84 of whom also underwent behavior and cognition testing and in 42 of whom FRAAs, TSH and FT4 were measured at prenatal and postnatal periods.[83] The results of this study suggest that variations in thyroid function do indeed have an effect on the behavior of children with ASD, although the exact mechanism for this influence in not clear. The authors suggest that it appears that fetal and neonatal exposure to blocking FRAAs could affect the development of the thyroid, potentially making the thyroid less sensitive to TSH.

## FLUORIDE ACCUMULATION IN THE PINEAL GLAND; MELATONIN DEFICIENCY AND AUTISM SPECTRUM DISORDER

Some symptoms of ASD, such as the sleep problems and the early onset of puberty, suggest abnormalities in melatonin physiology and dysfunctions of the pineal gland.[178] Luke[215] reported that fluoride accumulates in the pineal gland and that mongolian gerbils fed higher doses of fluoride excreted less melatonin metabolite in their urine and took a shorter time to reach puberty.[153] Luke analyzed the pineal glands from 11 human corpses, and found that the fluoride in the apatite crystals averaged about 9,000 ppm and in one case went as high as 21,000 ppm.[152] Melatonin is a hormone mainly synthesized in the pineal gland during the night. It is a biological signal of light/dark cycles and is considered as a major circadian synchronization system. Melatonin is responsible for regulating numerous life processes including reproduction, development, and aging. Melatonin has powerful neutralizing effects on ROS and LPP and increases the levels of several of the antioxidant enzymes in the brain. It is also an important modulator of mitochondrial metabolism, digestive functions, and immunity.

Decreased levels of melatonin in blood or urine have been reported as very frequent features in individuals with ASD compared to typically developing controls.[178] Many studies indicate clearly that nocturnal production of melatonin is reduced in ASD. Four studies have observed a correlation between abnormal melatonin concentrations and the severity of autistic behaviors. Babies with the lowest melatonin production had the most neurobehavioral problems.[185,260,266] The disruption of serotonin-N-acetylserotonin-melatonin pathway [Figure 11] has been suggested as a biomarker for ASD.[1,184] Pagan *et al*. assessed plasma melatonin and whole-blood serotonin in 278 patients with ASD, their 506 first-degree relatives (129 unaffected siblings, 199 mothers, and 178 fathers) and 416 sex- and age-matched controls.[184,185] They confirmed a deficit in melatonin in 51% (45–57%) as well as hyperserotonemia in 40% (35–46%) of ASD patients. Biochemical impairments were also observed in the first-degree relatives of patients.

## FLUORIDE AND NEUROINFLAMMATION: EVIDENCE FROM ANIMAL STUDIES

Few have examined the possibility of direct microglial activation by fluoride, despite that Shivarajashankara with colleagues reported in 2003 increased levels of ROS, RNS, and LPP in the blood of experimental animals treated with fluoride.[226] Wistar albino rats were exposed to 30 or 100 ppm fluoride in drinking water during their fetal, weanling and post-weaning stages of life up to puberty. An increase in brain LPP and ROS formation was found after the treatment of male adult Wistar rats with fluoride (10 ppm) for 30 days.[7] An increased immunoreactivity of glial fibrillary acidic protein was observed, which is specifically connected to astrocytes. Yan *et al*. reported that the supplementation of adult rats with high doses of fluoride (60 and 120 mg/L) in drinking water causes the activation of microglia in the hippocampus and cerebral cortex.[277] Fluoride initiates an inflammatory state through the synthesis of proinflammatory cytokines—IL-1B, IL-6, and TNF-α.

In astrocytes, fluoride activates phospholipase C (PLC) and increases intracellular $Ca^{2+}$ level. Once activated by fluoride, microglia secrete large concentrations of IL-1ß and TNF-α, which can then recruit more microglial activation in a vicious cycle that ultimately leads to neurodegeneration. Likewise, excitotoxins can activate microglia and stimulate release of inflammatory cytokines and additional glutamate.[240] Recently, Chen *et al*. reported that fluoride altered the inflammatory status and oxidative stress by inhibiting Wnt signaling pathway in BV2 microglial cells treated with various concentrations of NaF for 24 h. Wnt signaling has been implicated in developmental processes, in axonal remodeling, cytoskeletal organization, and neuronal plasticity.[47]

The abovementioned experiments suggest that the toxic effects of fluoride on the CNS may be attributed to the release of inflammatory cytokines and ROS, but fluoride concentration used in animal trials is very high. In humans, stimulation of the immune system with fluoride is not generally discussed among fluoride's adverse effects.

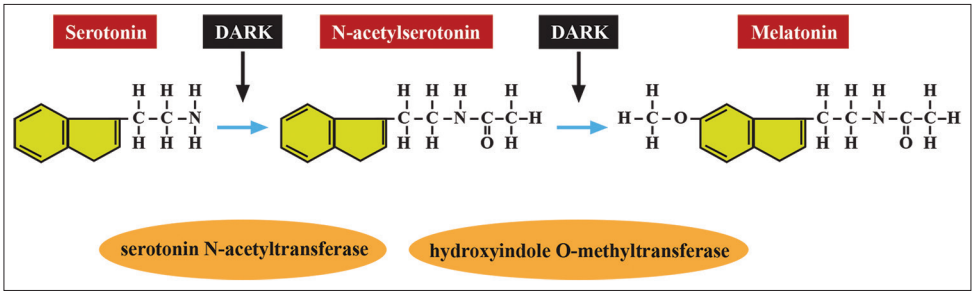


**Figure 11: Conversion of serotonin to melatonin. Serotonin is converted to melatonin through the action of two enzymes: serotonin N-acetyltransferase and hydroxyindole O-methyltransferase**

## ALUMINUM AS ENVIRONMENTAL TOXIC SUBSTANCE

$Al^{3+}$ has, until relatively recently, existed in forms not generally available to living organisms, and was therefore regarded as nontoxic. We underscore the remarkable and ongoing liberation of $Al^{3+}$ into the biosphere, resulting in its increased bioavailability to biological systems. The most significant factor driving complacency about the potential dangers of $Al^{3+}$ is its omnipresence in modern life. It is likely that $Al^{3+}$ is present in every physical and chemical compartment in the human body.[74,193]

However, $Al^{3+}$ is a non-essential element and it has no biological function in humans.

### Sources of aluminum; entry into body

There are a number of ways in which humans are exposed to $Al^{3+}$, such as through the skin, the lung, the nose, gastrointestinal system and of course, via intramuscular vaccination. Other major contributors include $Al^{3+}$ used in medicines: dialysis solutions, parental and intravenous nutrition solutions used in pediatrics. Vaccines, allergy skin tests, human serum albumin, baby skin creams, baby diaper wipes and antacids, which are frequently given to infants, are extremely high in $Al^{3+}$.

The primary natural source of $Al^{3+}$ is food, which provides approximately 16–100 fold more $Al^{3+}$ to systemic circulation than drinking water. While $Al^{3+}$ content of breast milk is very small (about 20 μg/L), a recent review of 30 infant formulas found that all contained $Al^{3+}$ and a number had concentrations far in excess of safety standards for oral consumption.[52] Impaired renal function as seen in infants and many elderly, greatly enhances the accumulation of $Al^{3+}$ in the body. It has been found that the mechanism for toxicity of $Al^{3+}$ is invariably biphasic with lower concentrations producing toxic effects through stimulatory actions and higher concentrations, resulting in inhibition of various physiological processes.[169,194]

### Aluminum as an activation agent of microglial-induced immunoexcitotoxicity

As a powerful immune activating element, $Al^{3+}$ can act both as the priming agent and the activation agent of microglial-induced immunoexcitotoxicity. This can occur with environmental exposure to excess $Al^{3+}$, as well as injection by vaccination. The major difference is that ingested $Al^{3+}$ is very poorly absorbed, whereas injected $Al^{3+}$ nanoaggregates with antibodies is completely absorbed and distributed throughout the body, including the brain.[90,126,199] A comprehensive review discussing the putative role of environmental $Al^{3+}$ in the development of chronic neuropathology in adults and children has been recently published by Morris, Puri, and Frye.[171]

### Aluminum-induced neurodegeneration

With the demonstrated widespread accumulation of $Al^{3+}$ in the brain and CSF following $Al^{3+}$ exposure, all the conditions for an intense and prolonged activation of brain inflammation are present. $Al^{3+}$ can activate microglia leading to secretion of TNF-α, IL-6 and cytokine-inducible NO synthase, and the development of neuroinflammatory ROS/RNS.[34,268,283]

**In animal models, aluminum leads to increased glutamate, potentiates damaging redox activity, displaces metal ions with strong binding capacity in cellular reactions, alters enzymes of oxidative metabolism in mitochondria, enters the brain, is active in developing brains, which could contribute to neurodegeneration.**

$Al^{3+}$ also induces significant alterations of glutamate/glutamine recycling within astrocytes leading to increased glutamine to glutamate conversion coupled with increased uptake of glutamate (see section 3).[244] $Al^{3+}$ through the potentiation of damaging redox activity or the disruption of intracellular $Ca^{2+}$ signaling can systematically disturb mechanisms of cellular defenses. In physiological tissues and compartments $Al^{3+}$ associates with oxygen donor ligands, but it prefers to bind with phosphates and displace normal biologically active metal ions. For example, $Al^{3+}$ binds almost $10^3$–$10^7$ times more strongly to ATP than does $Mg^{2+}$ and once $Al^{3+}$ acquires an energetically favorable electron-rich binding site, it has a tendency to remain there.[194]

$Al^{3+}$ alters the activity of several enzymes of oxidative metabolism in mitochondria, such as a significant decrease in the activity of cytochrome C oxidase, NADH and succinate dehydrogenase.[137,242] Impairment of mitochondrial energy metabolism in different regions of rat brain following chronic exposure to $Al^{3+}$ was reported by Kumar *et al*.[136] and Sharma *et al*.[217] The oxidative damage to mitochondria could play a significant role in $Al^{3+}$-induced neurodegeneration by a process of immunoexcitotoxicity. Several authors reported that $Al^{3+}$ intake caused a reduction of the activities of $Na^+$ $K^+$-ATPase and $Mg^{2+}$-ATPase, SOD, GPx and decrease levels of GSH in brain of rats.[171,196] Sharma and Mishra, using Wistar rats fed oral doses of aluminum chloride during gestation and postpartum periods, and found induced oxidative stress in the mother's brain as well as in the brain of the fetus and suckling mice, along with a decrease of GSH, GR, GPx, catalase, SOD, and acetylcholinesterase.[218]

In a study of the fate of injected $Al^{3+}$-adjuvants into muscle, researchers found that the $Al^{3+}$ particles were taken up mostly by microglia.[126] MCP-1/CCL2, a chemokine released from microglia upon activation, appears to be the main attractant for transfer of $Al^{3+}$ from the periphery to the brain.[40,126] As a post-mitotic organ, neurons and glia are especially vulnerable to $Al^{3+}$ toxicity and accumulation. As few as two injections of $Al^{3+}$-adjuvants in vaccine relevant doses was found to be sufficient to cause dramatic activation of rat brain

microglia and astrocytes that lasted up to 6 months post-injection. Such treatment leads to motor deficits and motor neuron degeneration.[10,219]

$Al^{3+}$ has no known beneficial physiological action in the human body. $Al^{3+}$ induced events likewise include oxidative stress, disruptions of energy metabolism, inflammation, glutamate excitotoxicity and effects on $Ca^{2+}$ homeostasis, and therefore might participate in etio-pathology of ASD.

## THE ROLE OF ALUMINOFLUORIDE COMPLEXES IN ETIOPATHOLOGY OF AUTISM SPECTRUM DISORDER

### Binding of aluminum to fluoride

The synergistic action of fluoride and $Al^{3+}$ has an important implication for pathological injury. Fluorine is the most chemically reactive nonmetal and the most electronegative element. $Al^{3+}$ binds fluoride anion more strongly than 60 other metal ions tested by Martin.[161] In water solution, $Al^{3+}$ forms, in the presence of fluoride, water soluble aluminofluoride complexes ($AlF_x$) whose average stoichiometry depends on an excess concentration of fluoride ions and the pH of the solution [for a review see: 240,242,243].

### Aluminofluoride as an analogue of phosphate groups in biochemical reactions

Under physiological conditions and even with micromolar concentrations of $Al^{3+}$, these two atoms react to form $AlF_4^-$, a molecule whose shape and physical properties closely resemble those of the phosphate anion, $PO_4^{3-}$. $AlF_4^-$ has been widely used as an analogue of phosphate groups to study phosphoryl transfer reactions and heterotrimeric G proteins involvement. Phosphoryl-transfer reactions are involved in processes such as regulation of cell metabolism, energy transduction, cytoskeletal protein assembly, regulation of cell differentiation and growth, aging, and apoptosis. Numerous laboratory studies demonstrated that $AlF_4^-$ interacts with all known G proteins and this feature has been exploited to help researchers understand phosphate-dependent reactions in signaling cascades.[93,197,204]

### Aluminofluoride binding to ADP and GDP b-phosphate and effects on protein conformation, interference in other signaling cascades

$AlF_4^-$ binds ionically to the terminal oxygen of ADP or GDP β-phosphate. ADP or GDP could therefore form a complex with $AlF_4^-$ that imitates ATP or GTP in its effect on protein conformation. This effect causes a structural change that locks the site and prevents the release of the γ-phosphate. Several authors have presented evidence that $AlF_4^-$ interferes with regulatory GTP hydrolases, which play an initiating role in regulation of effector enzymes.[103,272] The interactions of $AlF_4^-$ with signaling cascades of GPCRs have been documented by several authors using both biochemical as well as X-ray crystallographic analysis.

### Aluminofluoride and its influence on the immune system

Fluoride in the presence of micromolar $Al^{3+}$ affects the function of lymphocytes and cells of the immune system, ion transport, $Ca^{2+}$ influx and mobilization, protein phosphorylation, processes of neurotransmission, growth and differentiation, cytoskeletal proteins, and many other processes. These effects are not surprising when considering the extensive role of G proteins and GPCRs in the cell physiology. Approximately 800 GPCRs are encoded in the human genome. Physiological agonists include neurotransmitters and hormones, such as glutamate, dopamine, serotonin, melatonin, acetylcholine, TSH, neuropeptides, and excitatory amino acids.

$G_s$ proteins stimulate adenylyl cyclase (AC) to produce intracellular cyclic adenosine monophosphate (cAMP), whereas $G_i$ proteins inhibit the same process. Members of the cAMP-dependent second-messenger pathways participate in the regulation of cellular growth and differentiation and are also implicated in a variety of embryonic stages including brain development. cAMP is one of the key factors for neuronal outgrowth, plasticity, and regeneration.

### Animal studies on aluminum leading to autism spectrum disorder; suppression of melatonim

Notably, the autistic-like behaviors were observed in an animal model of adenylyl cyclase type 5 (AC5) knockout (KO) mice. Kim *et al*., reported that loss of AC5 in the dorsal striatum produces increased repetitive behaviors and sociability deficits.[128] Furthermore, pharmacological inhibition of mGluR3, GluA, and GluN, in the dorsal striatum in wildtype mice also induced autistic-like behaviors. Interestingly, group II mGluRs negatively modulate cAMP, explaining the link to melatonin suppression.[128,276] These results indicate that the inhibitory cAMP cascade is involved in the glutamate-evoked inhibition of melatonin synthesis and the authors suggested that it is likely that the glutaminergic system functions as an autonomic regulatory mechanism against neuronal control in the pineal gland.

### In animal models aluminofluoride effects metabolic processes governing $Ca^{2+}$ regulation that can affect excitotoxicity, can lead to neurodegeneration; low dose fluoride enhances uptake of aluminum, fluoride in tap water likely fed to infants; Aluminofluoride complex can lead to neurodegeneration

Many of metabolic and physiological processes are affected by alterations in intracellular $Ca^{2+}$ levels.

$G_{\alpha q}$ mediate phospholipase C (PLC)-dependent phosphoinositide hydrolysis, yielding inositol-1,4,5-trisphosphate and diacylglycerol (DAG) as second messengers to trigger both the increase of intracellular $Ca^{2+}$ level as well as PKC activation [Figure 5]. Dysregulation of homeostasis of $Ca^{2+}$ in the cell plays a major role in excitotoxicity. $AlF_4^-$ mimics the effects of $Ca^{2+}$ mobilizing hormones by activating the G protein, which couples the hormone receptor to PLC. $AlF_4^-$ can evoke the disturbance of $Ca^{2+}$ homeostasis, which markedly affects brain development and functions.[240]

It has been, for example, observed that $Al^{3+}$-induced neural degeneration in rats is greatly enhanced when the animals were fed low doses of fluoride. The presence of fluoride caused more $Al^{3+}$ to cross the BBB and be deposited in the brain of rats. The formation of $AlF_4^-$, according to experimental evidence, in quantities as little as 1 ppm of fluoride contamination of water supplied to rats led to a greater uptake of $Al^{3+}$ into the brain along with the formation of amyloid deposits like those in Alzheimer's disease.[218,265] Strong evidence exists that fluoride could complex with any pre-existing $Al^{3+}$ within body fluids to produce $AlF_4^-$. With over half of mothers using infant formula reconstituted using tap water, infant exposure to significant levels of $AlF_4^-$ might become an important risk factor in etiopathology of ASD.

## Amplification of false message of $AlF_4^-$ in signaling cascades

The effects of $AlF_4^-$ are amplified by processes of signal transduction [Figure 12]. The principle of amplification of the initial signal during its conversion into a functional response has been a widely accepted tenet in cell physiology. It is evident that $AlF_4^-$ is a molecule giving a false message. As G-protein coupled receptors (GPCRs) lie upstream of processes that produce key intracellular second messengers, it does not come as a surprise that the false signal of $AlF_4^-$ has the potential to modulate neurodevelopment, brain structure, structural plasticity, as well as higher cerebral functions.

Basically, by mimicking the mechanism that activates G-protein signaling, aluminofluoride can activate several reactions utilizing G-protein signaling, such as metabotropic glutamate receptors. Protein kinases are essential for GPCRs-mediated signal transduction. $AlF_4^-$ mimics the transition state of protein phosphorylation. Protein phosphorylation is also important in posttranslational modification of GluRs. The studies of mGluRs show that like many GPCRs they can activate more than a single G protein, can produce various second messengers, and interact with several common protein kinases. The effects of $AlF_4^-$ might thus result in numerous pathophysiological consequences at several times lower concentrations than either $Al^{3+}$ or fluoride acting alone. Several common protein kinases, including protein kinase A (PKA), PKC, and mitogen-activated protein kinases (MAPKs), directly interact with mGluR1/5, phosphorylate specific serine or threonine sites, and thereby regulate trafficking, distribution, and function of mGluR1/5 receptors.[141]

Ji *et al.*, examined alterations in the activity of cAMP-dependent PKA (Protein Kinase A) and PKC (Protein Kinase C) in postmortem brain tissue samples from the frontal, temporal, parietal, and occipital cortices, and the cerebellum of individuals with regressive autism as compared to age-matched control subjects and autistic subjects without clinical history of regression.[117,118] (For example, Through a series of steps, Protein Kinases lead to the phosphorylation of amino acids and molecules changing their activity leading to other molecular

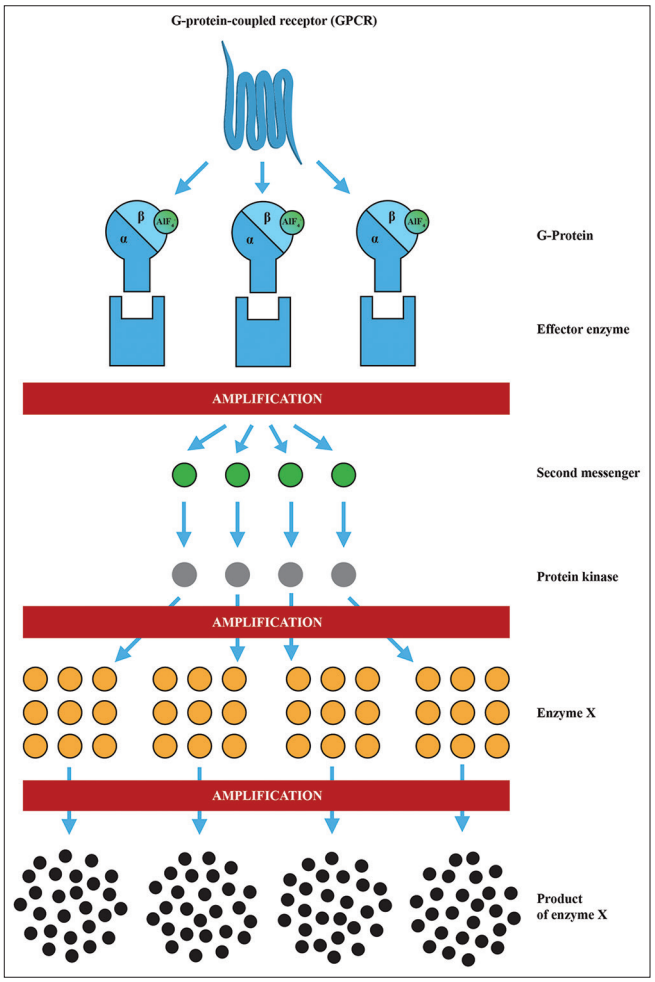


**Figure 12: $AlF_4^-$ acts as the messenger of false information. Its message is greatly amplified during the conversion into the functional response of a cell. Effector enzymes are adenylyl cyclase or phospholipase C, the second messenger molecule could be cAMP, inositol 1,4,5-trisphosphate and diacylglycerol**

steps.) Neuronal tissues normally have high activity of PKC, but these authors found that PKC activity was significantly decreased by 57.1% in the frontal cortex of subjects with regressive autism, as compared to age-matched control subjects and by 65.8% as compared to non-regressed autistic subjects. The activity of PKC might also reflect the stimulation of the group I mGluRs, which enhances excitotoxicity. These authors suggested that regression in autism may be attributed, in part, to alterations in GPCR-mediated signal transduction involving PKA and PKC in the frontal cortex and that it is possible that both PKC and PKA are affected by a common pathway. According to our explanation, the alterations in protein kinases in some parts of brain in subjects with regressive autism might reflect an intoxication with $AlF_4^-$. The involvement of PKC has also been reported in other conditions such as inflammation, immune disorders, and oxidative stress, which are reported by several authors as the hallmarks of ASD.[45,75,206]

In another trial, Akinrinade *et al*., treated male adult Wistar rats with low and high doses of fluoride, $Al^{3+}$ or a combination of both elements for 30 days and compared their effects on brain homogenates.[7] Both $Al^{3+}$ and fluoride activated astrocytes, with the greatest activation seen with the high dose $Al^{3+}$. They observed significant oxidative stress and high levels of lipid peroxidation products (LPP). Even low dose exposure of these two elements can result in neurotoxic effects. Taken together, the results may demonstrate a close link between oxidative stress, neuroinflammation, and $AlF_4^-$ induced toxicity.

In recent years, our understanding of function of G-protein coupled receptors (GPCRs) has changed from a picture of simple signal relays, transmitting only a particular signal to a particular G protein, to versatile machines, capable of various responses to different stimuli and being modulated by various factors. Malfunction of myriads of GPCRs functions is prevalent in human diseases, so that approximately half (estimates vary between 30 and 60%) of marketed drugs target GPCRs.[141]

The synergistic action of fluoride and micromolar $Al^{3+}$ can thus evoke a whole network of pathological events. Simultaneously, the heterogeneity of their mutual dynamic interactions can explain the clinically heterogeneous symptoms of ASD and contribute to an understanding of the various responses in any given child to the identical environmental neurotoxins, since we know that each case of autism is unique.

## PREVENTION AND AMELIORATION OF AUTISM SPECTRUM DISORDER SYMPTOMS

Recently, Lyra *et al*. published a review called *What do Cochrane systematic reviews say about interventions for* autism *spectrum disorders?*[154] They evaluated seventeen reviews and found weak evidence of benefits from acupuncture, gluten and casein-free (GFCF) diets, early intensive behavioral interventions, music therapy, parent-mediated early interventions, social skill groups, Theory of Mind cognitive model, aripiprazole, risperidone, and tricyclic antidepressants. No benefits were found for sound therapies, chelating agents, hyperbaric oxygen therapy, omega-3, secretin, vitamin $B_6/Mg^{2+}$ and SSRI for children.[154] These findings show that despite intensive research the effective interventions for amelioration of ASD symptoms supported by evidence-based medicine have not yet been proved. Unfortunately, there was no effort to differentiate between the infantile form of autism and the postnatal form, as the severity of the neurological deficits and degree of disorganized brain development, can vary greatly.

## HYPOTHESIS FOR THE DEVELOPMENT OF AUTISM SPECTRUM DISORDER FROM AVAILABLE EVIDENCE AND POSSIBLE METHODS OF REVERSAL OF THE SYMPTOMATIC EXPRESSION OF AUTISM SPECTRUM DISORDER AFFECTED NERVOUS SYSTEM SUPPORTED BY OBSERVATIONS FROM THE LITERATURE

Based on our hypothesis of immunoexcitotoxicity we suggest that it is reasonable to conclude that infants and children are exposed to a number of environmental and food-based excitotoxin additives, or conditions that can worsen intrinsic brain inflammation and excitotoxicity. What has emerged is the insight by most investigators that ASD is a complex, multisystem disorder. Disruptions in energy metabolism resulting in mitochondrial dysfunction, impairment in critical regulation of oxidative stress with disturbances of transmethylation/transsulfuration system and decreased GSH level, produce a systemic disorder that affects brain development and function. Basic to this hypothesis is the finding that one sees extensive and prolonged microglial and astrocytic activation and resulting inflammation/excitotoxicity in most cases of ASD. In this review, we show that processes of immunoexcitotoxocity are both triggered and amplified by the environmental toxin fluoride and $Al^{3+}$.

Reversal of fluoride induced cell injury and fluorosis through the elimination of fluoride and consumption of a diet containing essential nutrients and antioxidants, have been shown by studies in India to be beneficial to brain function, where millions of people suffer with fluorosis.[245] Concerns of Nobel Laureate in Medicine Arvid Carlsson about what increased fluoride levels in drinking water would do to the developing brain of

newborn infants have gained renewed significance in light of recent findings concerning fluoride neurotoxicity and fluoride ability to prime/activate microglia.[256]

## Role of nutrition in brain development and the role of Vitamins C, E, and $D_3$, as antioxidants, in reversing the metabolic changes found in fluoride intoxication

Early nutrition has been shown to play a major role in the brain development, mental and immune function. Children and adolescents with poor nutritional status are exposed to alterations of mental and behavioral functions that can be corrected to a certain extent by dietary measures.[36,257,275] Many of the vitamins used to treat fluoride intoxication and ASD are antioxidants, which can significantly reduce excitotoxicity. Ameliorative effects of vitamins C, E, and $D_3$ alone, and in combination, were intensively studied in laboratory animals.[49] Vitamins C and E act as antioxidants scavengers of free radicals and peroxides, which accumulate after fluoride exposure. Vitamin E channels the conversion of GSSH to GSH, which in turn helps conversion of mono- and dehydroascorbic acid to maintain ascorbic acid levels. Oral administration of vitamin C (50 mg/kg body weight/day) and vitamin E (2 mg/0.2 ml olive oil/animal/day) from day 6 to 19 of gestation along with NaF (40 mg/kg body weight) significantly ameliorated NaF-induced total percentage of skeletal and visceral abnormalities in rats.[267] Vitamin E was comparatively less effective than vitamin C. Co-treatment with vitamins C, $D_3$, and E ameliorates NaF-induced reduction in serum $Ca^{2+}$ and phosphorus.[70,100] Toxic effects of fluoride were reversible if its exposure was withdrawn for two months. Recovery was also possible by feeding antioxidants (SOD, GSH, β-carotene, and some herbal extracts).[48,146] A single trial exploring the effectiveness of ascorbic acid (8g/70kg/day) as a supplemental therapy for autism reported a reduction in symptom severity.[65] Trials exploring the effect of vitamin C in subjects with ASD have not been repeated up until today, but the case reports of scurvy in autistic children are emerging.[156,192]

**Table 2: Prevention and amelioration of ASD symptoms**

| Special supplements and compounds, which are known to reduce | Excitotoxicity, oxidative stress, and microglial activation |
|---|---|
| Pyridoxal-5 phosphate (vitamin $B_6$) | Magnesium glycinate, lactate or threonate |
| Methylcobalamin (vitamin $B_{12}$) | Zinc |
| Folic acid, folate (vitamin $B_9$) | Silicon-rich mineral waters |
| Tri- or Dimethylglycine (vitamin $B_{15}$) | Melatonin |
| Vitamin E (mixed tocopherols) | GSH |
| Vitamin C (buffered) | Taurine |
| Vitamin D3 | Tryptophan |
| Coenzyme Q10 | Flavonoids - quercetin |
| Acetyl-L-carnitine | Baicalein |
| Alpha-lipoic acid | Resveratrol |
| DHA/EPA- omega3 fatty acids | Curcumin |

## Role of vitamin $B_6$ and vitamin $B_{12}$, in treating immunoexcitotoxicity

Vitamin $B_6$ can dramatically lower blood and tissue glutamate levels and raise seizure thresholds. High dose of vitamin $B_6$'s ability to powerfully inhibit excitotoxicity at least partially explains the often dramatic results reported by Rimland in treating autistic children.[201] Vitamin $B_6$ has also been shown to lower brain and blood glutamate levels and to raise GABA levels. In addition, along with folate and vitamin $B_{12}$, it reduces homocysteine levels [Figure 9]. Methylcobalamin is a GluR blocker and supplementation with it improves cerebral and cognitive functions.[6,36] Alterations in processes of methylation and transsulfuration have been studied in detail in ASD. James and her colleagues were able to fix the biochemical disruptions through nutritional supplementation with vitamins $B_6$, folinic acid (reduced form of folate – $B_9$), $B_{12}$, and pangamine ($B_{15}$).[115] Frye and Rossignol[82] investigated in double-blind, placebo-controlled studies l-carnitine and a multivitamin containing B vitamins, antioxidants, vitamin E, and Co-enzyme Q10 while non-blinded studies have investigated ubiquinol. Controlled and uncontrolled studies using folinic acid have reported marked improvements in core and associated ASD symptoms in some children with ASD.

## Role of vitamin $D_3$ in treating autism spectrum disorder

Vitamin $D_3$ deficiency is accounted among the potential environmental risk factors for ASD.[179] Oral supplementation with vitamin $D_3$ is popular among interventions for ASD considering its relative safety and cheapness. An enormous body of scientific literature considering the mechanism of vitamin $D_3$ effects led to a new view on its role in human health. The metabolites of vitamin $D_3$ act as a hormones, regulating a plethora of functions and processes in human body.[129,181]

Saad *et al.* published a controlled RTC study on 122 ASD children and found that 57% of the patients had vitamin D deficiency, and 30% had vitamin D insufficiency.[208] The children received vitamin $D_3$ (300 IU/kg/day) for 3 months. Collectively, 80.72% (67/83) of patients who received vitamin $D_3$ treatment had significantly improved outcome, which was mainly in the sections of the CARS and aberrant behavior checklist subscales that measure behavior, stereotypy, eye contact, and attention span. While this study showed that oral supplementation with vitamin $D_3$ may help improve some symptoms of ASD, Kerley *et al.* reported that on a group of 42 children with ASD in Ireland, vitamin $D_3$ supplementation had no effect on the primary outcome with limited and inconsistent

effects.[124] The children in this study received 2000 IU vitamin $D_3$ supplementation or placebo daily for 20 weeks. Cieslinska *et al*. studied vitamin D receptor gene polymorphisms associated with ASD.[54] This team found that vitamin $D_3$ serum levels were not significantly different between ASD and non-ASD children and concluded that this could indicate that the functionality of vitamin $D_3$ metabolism might be affected and should be considered when studying the development of symptoms among ASD children.

## Role of diets in autism spectrum disorder

Many of the diets now being proposed for autistic children emphasize elimination of foods known to be exceedingly high in excitotoxin additives. They are also low in sugar. Jones *et al*. demonstrated that children respond to glucose challenges with a hypoglycemic response at higher levels of blood glucose than adults.[119] Autistic children have altered energy metabolism and a high incidence of reactive hypoglycemia, which increases their risk of seizures and excitotoxicity.[53]

## Other neutraceuticals that may be of benefit in treating autism spectrum disorder

A number of nutraceuticals have been shown to improve mitochondrial function, including thiamine, riboflavin-5 phosphate, pyridoxal-5 phosphate, ascorbate, acetyl-L-carnitine, pyruvate, malate, CoQ10, curcumin, niacinamide, ketones, DHA, vitamins K, folate and methylcobalamin.[64,99]

Taurine may play a crucial role in the protection of antioxidant system and ATPases against $Al^{3+}$-induced toxicity in brain and blood of rats. Deficiency of taurine in a cat leads to immune activation in the CNS with Purkinje cell loss, microglial activation and astrocytosis, similar to that observed in the ASD brain.[188] Taurine plays an important role in development of the eye and brain, reproduction, immune function, including anti-inflammatory activity, anti-oxidant activity, membrane stabilization and osmoregulation. Taurine concentrations in blood of ASD children have been determined in several laboratories. However, the reports are controversial, with some studies showed lower, no change or higher taurine concentrations in autistic groups compared to controls.[89,188] Nevertheless, Park *et al*. hypothesize that taurine can ameliorate the development of ASD when delivered at the appropriate time and dosage due to its effects on abnormalities of the innate immune system and oxidative stress.[188]

Many parents of children with ASD report that their child has stool with a sand-like appearance. One of the most common causes comes from a lack of bile acid formation in the liver. Taurine conjugates with bile acids and increases their pool size. Some autism intervention specialist therefore recommend supplementation with taurine.

There is a need for a non-invasive method to both reduce the absorption of $Al^{3+}$ in the gastrointestinal system and facilitate the excretion of systemic $Al^{3+}$ in the urine, especially in children, pregnant mothers and women of childbearing age who may become pregnant. Based on the knowledge that silicon is the natural antagonist to $Al^{3+}$, some researchers have shown that silicon-rich mineral waters can be used to reduce the body burden of $Al^{3+}$ in individuals with Alzheimer's disease, macrophagic myofasciitis, and chronic fatigue syndrome.[61,211]

Pogue and Lukiw[229] reported that drinking up to 1 L of a silicon-rich mineral water each day for 12 weeks facilitate the removal of $Al^{3+}$ via the urine without any concomitant affect upon the urinary secretion of iron and copper.[194] There is some evidence that silicone could act either as a neuroprotector, since low doses increased cell viability and reduced TNF-α levels, or as a neurotoxic agent at higher concentrations.[86]

$Mg^{2+}$ and zinc also powerfully inhibit excitotoxicity as well as act as co-factors in numerous enzyme systems. Low $Mg^{2+}$ is associated with dramatic increases in free radical generation as well as GSH depletion. In addition, low $Mg^{2+}$ enhances NMDA sensitivity, making excitotoxicity more likely even in the presence of physiological levels of extracellular glutamate. High glutamate levels have also been shown to deplete cellular GSH.

Of great interest is the use of selected flavonoids as antioxidants, anti-inflammatories, GluR blockers, and antimicrobials. The flavonoids are more powerful and versatile as antioxidants than are the vitamins.[30] A number of plant polyphenols have been shown to reduce microglial activation and reduce injury by immunoexcitotoxicity, including curcumin, quercetin, silymarin, DHA, green tea catechins, resveratrol, baicalein, apigenin, luteolin, butyrate and wogonin[32] [Table 2].

Supplementation with the amino acid tryptophan is considered in the treatment of depression and sleep disorders, mainly due to its relationship with the synthesis of serotonin and melatonin. It is also used in helping to resolve cognitive disorders, anxiety, or neurodegenerative diseases.[120] It should be appreciated that under inflammatory conditions, metabolism of tryptophan is shifted to the production of a rather powerful excitotoxin, quinolenic acid, mainly by the induction of the enzyme indoleamine-2,3-dioxygenase (IDO).

In August 2017, Li with co-workers[139] reviewed evidences of carefully selected 22 randomized controlled trials on 8 different dietary supplements for core symptoms of ASD.[145] Among the supplements reviewed, three (methyl $B_{12}$, vitamin $D_3$, and omega-3 fatty acids) were guided by deficiencies in ASD; three (folinic acids, probiotics, and CFGF diet) were guided by potential etiological theory, and two (camel milk and sulforaphane) were guided by

anecdotal evidence. However, the authors concluded that most studies were small and short term, and there is little evidence to support effectiveness of dietary supplements for children with ASD.

### Value of melatonin in the treatment of autism spectrum disorder

Rossignol and Frye evaluated twenty clinical studies, which have reported improvements in sleep parameters with exogenous melatonin supplementation in ASD, including longer sleep duration, less nighttime awakenings and quicker sleep onset.[206] A meta-analysis of five randomized, double-blind, placebo-controlled crossover trials examining exogenous melatonin supplementation in ASD reported significant improvements with large effect sizes in total sleep duration and sleep onset latency compared to both baseline and placebo. Six studies reported that the nighttime administration of exogenous melatonin was associated with better daytime behaviors. Four studies reported improvements with exogenous melatonin supplementation when other sleep medications had previously failed. Adverse effects of melatonin were minimal to none in the twenty treatment studies.[206] These studies indicate that the administration of exogenous melatonin for abnormal sleep parameters in ASD is evidence-based. Since the production of melatonin is maximal in dark, infants and children would sleep in a dark room during the night to induce the physiological melatonin circadian rhythm.

## CONCLUSIONS

The etiology and pathogeneis of ASD are not well understood. Autism Spectrum Disorders comprise a complex of clinical syndromes found predominantly in infants and younger people consisting of disturbances in cognition and comprehension, communication disorders, epilepsy, and behavior that have an underlying common pathology of neurodegeneration of the cerebellar Purkinje cells and other areas of the brain. Other studies in humans have shown chronic inflammatory changes in the brain, particularly in the cerebellum, and involvement of the microglia in this pathology.

Our review presents evidence suggesting a hypothesis unifying the syndromes in ASD. The clinical as well as pathological findings of the ASD have a set of pathological events with the common denominator being immunoexcitotoxicity leading to neurodegeneration and abnormalities in the connectome, particularly in developing brains in the neonate and young with evidence that explains the clinical presentation of ASD.

Our hypothesis is supported by experimental evidence from animal models and by some clinical testing and pathology studies that give credibility to the concept of immunoexcitotoxicity as the underlying cause of ASD. A great number of conditions can trigger both the inflammatory and the excitotoxic cascade, including sequential vaccination, infections, hypomagnesemia, ROS, RNS and LPP, fluoride and $Al^{3+}$ as well as a number of other neurotoxic metals and industrial chemicals. Chronic activation of the brain's immune system increases extracellular glutamate levels sufficiently to trigger the excitotoxic cascade, which in conjunction with inflammatory cytokines and prostaglandins, magnifies the damaging effects of both. This mechanism explains most of the features of the ASD, including the behavioral difficulties, language problems, repetitive behaviors, intellectual delay, and episodic dyscontrol of anger. In addition, these mechanisms explain the pathological findings as well, including the changes in the cerebellum, abnormalities in connectivity and the widespread activation of microglia and astrocytes. It also explains why ASD has not disappeared despite the removal of mercury from most childhood vaccines, since excessive immune activation is the initiating and sustaining event in ASD. Evidence is presented that the abundance of fluoride added to the water worldwide and the widespread availability of aluminum particularly to infants and young children through aluminum containing vaccinations, singly or together as aluminofluoride can be potent factors in producing the condition of immunoexcitotoxicity that leads to the pathological changes seen in ASD. The vaccination program should be evaluated to reduce the excessive stimulation of immature immune system and to replace $Al^{3+}$-adjuvants.

We have also reviewed studies that indicate that fluoride and $Al^{3+}$, as ubiquitous environmental and food-derived toxins, can exacerbate the pathological and clinical problems of ASD. In synergistic action as $AlF_4^-$ these elements induce numerous chronic pathophysiological consequences at several times lower concentrations than either $Al^{3+}$ or fluoride acting alone. $AlF_4^-$ may evoke several signaling disorders and act as an endocrine disruptor. Moreover, most of the excitotoxic events may enhance the subclinical pathological alterations and/or the genetic susceptibility seen in ASD. The full genetic potential of the child for brain and mental development may be also compromised due to deficiency of micronutrients.

Our immunoexcitotoxic hypothesis opens the door to a number of new modes of prevention and amelioration of ASD. Elimination of various sources of fluoride and $Al^{3+}$ in early development and consumption of a diet containing essential nutrients and antioxidants have been shown to be beneficial to brain function. As a multifaceted disorder, ASD requires a multifaceted approach, one that should include the protection against excitotoxicity, as well as the protection against microglial activation.

There are several experimental studies that can be constructed to test the immunoexcitotoxic hypothesis, especially as regards vaccines. Such a study would require the use of non-human primates at various stages of development, from intrauterine life to early post-natal development corresponding to human neurodevelopment during vulnerable neurodevelopmental milestones. The study would follow the vaccine schedules used with human newborns and pre-schoolers. One would need to use the exact vaccines used in the human vaccine schedules but of comparable doses based on weight.

To measure microglial activation in response to the vaccines, it would be necessary to use microglial activation PET scanning techniques at various time schedules before and following the vaccines. Newer microglial activation PET scanning techniques are being developed that hold the possibility of differentiating between M1 and M2 microglial activation phenotypes. Long-term follow-up scanning would be necessary to delineate prolonged microglial activation as has been observed in cases of autism. Measure of glutamate levels in the affected areas of the brain should also be conducted, perhaps in a separate set of monkeys to prevent inadvertent activation of local microglia. More extensive studies could be envisioned, such as measures of EAATs, glutaminase, and NADPH oxidase within affected brain areas in response to vaccination.

In addition, studies should be conducted measuring the aluminum, fluoride and aluminofluoride complex concentration in the affected areas of the brain in autopsied cases of autism. This should include the various cell types as well as cellular compartments. This could also be done experimentally in the non-human primate studies using human vaccines described above. Controls could establish the levels of these toxicants to establish the baseline levels in non-vaccinated animals.

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